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NATURAL KILLER CELLS, MULTIPLE MYELOMA, AND DARATUMUMAB
- A LOVE-HATE RELATIONSHIP

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NATURAL KILLER CELLS, MULTIPLE MYELOMA, AND DARATUMUMAB
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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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"I've gone on record to say that by 2025, cancer researchers will have developed curative therapeutic approaches for most if not all cancers."

Gary Gilliland

President and Director, Fred Hutchinson Cancer Research Center (FHCRC)

2018

ABSTRACT

Multiple myeloma is a treatable, but not yet curable, malignant plasma cell disease. Over the last decades, the overall survival time of multiple myeloma patients has constantly increased thanks to improvements in conventional treatment methods like chemotherapy as well as autologous stem cell transplantation both in combination with novel drugs such as proteasome inhibitors or immunomodulatory drugs. However, no cure has been found yet. The only treatment that has the potential to cure multiple myeloma is allogeneic stem cell transplantation. However, it is seldomly used due to lack of donor availability, high risk for treatment related mortality and occurrence of graft versus host disease.

The development of monoclonal antibody treatments for several cancer indications has shown great success. Lately, the emergence of Daratumumab, an anti-CD38 monoclonal antibody targeting plasma cells, has given a new hope for patients. Daratumumab has a minor direct effect on the MM cells but the majority of its effectiveness lies in utilizing the patient's own immune cells to find and clear the body from MM cells. Several immune cells are known to be involved in this process such as natural killer cells, T cells and macrophages.
Natural killer cells are experts in detecting virus infected or malignant transformed cells without the need for prior activation, and they are at the forefront of immune response against malignant cells and thus a promising option for cancer immunotherapy.

In study I we could show that two heavily pretreated, triple refractory multiple myeloma patients, who received Daratumumab treatment and progressed, could be re-challenged with the same drug. We observed that the recurring multiple myeloma cells showed normal CD38 expression after a short treatment interruption. This made those patients eligible for a second line of Daratumumab treatment which has proven to be save. In both patients a partial response could be observed. Additionally, we reported that natural killer cells were depleted immediately after Daratumumab administration.

The lack of natural killer cells in Daratumumab treated patients leaves them at risk for viral reactivation or bacterial infection. In study II we observed that an unusually high percentage of Daratumumab treated patients suffered from infectious complications, of which viruses of the herpes family were the most prominent. We monitored the immune status of those patients and their clinical parameters and one of our observations was that natural killer cells were reduced in general, but in particular the more mature natural killer cell population was depleted.
These findings led us to the conclusion that combining monoclonal antibody treatment with adoptive cell transfer may have a synergistic effect and allow for better disease control.

Producing natural killer cells in large quantities for clinical trials is challenging. One pivotal factor for robust cell expansion is serum, which is an undefined component with big batch to batch variation that will have a big impact on expansion rates und functionality of the cells. In order to circumvent this problem, we adapted the clinically used natural killer cell line NK-92 to serum-free conditions with inherited phenotype and growth rate.

Additionally, we reported that serum-free NK-92 cells showed elevated functionality towards K562 cells after reintroduction of serum. We also performed RNA sequencing to compare serum-free cultured NK-92 cells with cells cultured under standard conditions to investigate the biological mechanisms involved in serum reduction.

Altogether we propose that growing serum-free NK-92 cells is feasible and the reported protocol is robust, cheap and can be adapted for clinical grade production. Whether the combination of these cells with other advanced treatments will show additive or synergistic treatment outcomes for multiple myeloma patients, needs to be evaluated in future studies.

LIST OF SCIENTIFIC PAPERS

I. Alici E, **Chrobok M,** Lund J, Ahmadi T, Khan I, Duru AD, Nahi H[2]

Re-challenging with anti-CD38 monotherapy in triple-refractory multiple myeloma patients is a feasible and safe approach.
Br J Haematol. 2016 Aug;174(3):473-7.
DOI: 10.1111/bjh.13776.

II. Hareth Nahi, **Michael Chrobok**, Charlotte Gran, Johan Lund, Astrid Gruber, Gösta Gahrton, Per Ljungman, Arnika K. Wagner, Evren Alici[2]

Infectious complications and NK cell depletion following daratumumab treatment of Multiple Myeloma.
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III. **Michael Chrobok**[1], Carin I.M. Dahlberg[1], Ece Canan Sayitoglu, Vladimir Beljanski, Hareth Nahi, Mari Gilljam, Birgitta Stellan, Tolga Sutlu, Adil Doganay Duru, Evren Alici[2]

Functional Assessment for Clinical Use of Serum-Free Adapted NK-92 Cells.
Cancers (Basel). 2019 Jan 10;11(1). pii: E69.
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CONTENTS

LIST OF ABBREVIATIONS

Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
ADCP	antibody-dependent cellular phagocytosis
allo-SCT	allogeneic stem cell transplantation
AML	acute myeloid leukemia
APC	antigen presenting cell
ASCT	autologous stem cell transplantation
BAT3	HLA-B-associated transcript
BCMA	B cell maturation antigen
BM	bone marrow
BMSC	bone marrow stromal cell
Bort	Bortezomib
CAR	chimeric antigen receptor
Carf	Carfilzomib
CDC	complement dependent cytotoxicity
CGMP	current good manufacturing practice
CLL	chronic lymphocytic leukemia
CR	complete response
CRACC	CD2-like receptor activating cytotoxic cell
CRP	C-reactive protein
CRS	cytokine release syndrome
Dara	Daratumumab
DC	dendritic cell
Dex	Dexamethasone
DNAM-I	DNAX Accessory Molecule-I
ELd	Elo with Len and Dex
Elo	Elotuzumab
EMA	European Medicines Agency
ER	endoplasmatic reticulum
EV	extracellular vehicle
FasL	factor ligand superfamily
FDA	Food and Drug Administration
GM-CSF	granulocyte macrophage colony-stimulating factor
GvHD	graft versus host disease
HCMV	humane cytomegalovirus
HLA	self-human leukocyte antigen
HSC	hematopoietic stem cell
HSV	herpes simplex virus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMiD	immunomodulatory drug
kDa	kilodalton

KIR	killer immunomodulatory-like receptor
Ld	Len and Dex
mAbs	monoclonal antibody
MCMV	murine cytomegalovirus
MCP	monocyte-recruiting chemotactic protein
MDSC	myeloid-derived suppressor cell
MGUS	monoclonal gammopathy of undetermined significance
MHC	major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
MIL	bone-marrow infiltrating lymphocyte
MM	multiple myeloma
MP	melphalan and prednisone
NCR	natural cytotoxicity receptor
NHL	non-Hodgkin lymphoma
NK	natural killer
NTB-A	natural killer, T and B cell antigen
OS	overall survival
PBMC	peripheral blood mononuclear cell
PD-LI	PD1-ligand
PFS	progression-free survival
PI	proteasome inhibitor
Pom	Pomalidomide
PR	partial response
PVR	poliovirus receptor
RRMM	relapsed/refractory MM
SD	stable disease
SLAM	signaling lymphocytic activation molecule
SLAMF	signaling lymphocyte activation molecule-related receptor family
sMIC	soluble MIC
SMM	smoldering multiple myeloma
TAM	tumor-associated macrophage
TCR	T cell receptor
TGF	tumor growth factor
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
UPP	ubiquitin-proteasome pathway
WBC	white blood cell counts
VGPR	very good partial response
VZV	varicella-zoster virus

1 INTRODUCTION

1.1 MULTIPLE MYELOMA

Multiple myeloma (MM) is a malignant neoplasm of terminally differentiated, immunoglobulin (Ig)-producing, long-lived plasma cells. The hallmark feature of MM is the monoclonal expansion of plasma cells in the bone marrow with accompanying excessive production of monoclonal Ig that can be detected as a big spike in protein electrophoresis in the gamma zone coming from massive myeloma protein production; this spike is called "M spike" (Raab, Podar et al. 2009). MM is more prevalent in elderly population; the median age is 72 years in Sweden. The amount of heterogeneous chromosomal aberrations and numerous mutations in several genes are among the key elements of this disease; therefore MM is difficult to target therapeutically (Morgan, Walker et al. 2012).

MM can start with an asymptomatic premalignant lesion stage that is called monoclonal gammopathy of undetermined significance (MGUS), progresses further to smoldering multiple myeloma (SMM) and eventually becomes symptomatic MM. The last step is also characterized by bone marrow (BM) infiltration and osteolytic lesions (Fairfield, Falank et al. 2016). Typical symptoms of BM are net bone loss due to the skewing of the equilibrium between bone building osteoblasts and bone resorbing osteoclasts as well as increased fracture risk (Fowler, Edwards et al. 2011, Drake 2014). The signs for MM are often described by the acronym CRAB (elevated calcium, renal insufficiency, anemia, bone disease) while the transition between MGUS, SMM and MM is fluent and usually classified as having high serum or urinary monoclonal protein as well as 10-60% clonal plasma cells in the BM (Ghobrial and Landgren 2014, Rajkumar, Dimopoulos et al. 2014, Glavey and Ghobrial 2015).

MM cells very often have mutations in the RAS family (KRAS, NRAS, BRAF) or TP53 and DIS3 (Lohr, Stojanov et al. 2014). In many cases, the loss of the short arm of chromosome 1 (1p) and inactivation of p53 results in an abundance of Ig production (Furukawa and Kikuchi 2015). Chromosomal aberrations are also very common in MM. Several subclones with different chromosomal aberrations can be found within the same patient and these subclones will most likely develop early on in the disease progression and might be responsible for relapse (de Mel, Lim et al. 2014, Prideaux, Conway O'Brien et al. 2014, Corre, Munshi et al. 2015). The Ig heavy chain gene is dominating in the most common chromosome translocations in MM such as: t(11;14), t(4;14), t(6;14), t(14;16), t(14;20) and the most abundant chromosomal gains and losses are: gain of 1q, loss of 1p, loss of 13/13q and loss of 17p (Anderson and Carrasco 2011, Prideaux, Conway O'Brien et al. 2014).

1.2 NATURAL KILLER CELLS

Cells that showed cytotoxic reactions to leukemia-associated antigens without previous sensitization were first reported by Rosenberg *et al.* in 1972 (Rosenberg, Herberman et al.

1972). Just 3 years later, the same cells were discovered by Rolf Kiessling and Eva Klein, who gave them their name natural killer (NK) cells; in parallel, researchers around Ronald Herberman discovered the same cell type (Herberman, Nunn et al. 1975, Herberman, Nunn et al. 1975, Kiessling, Klein et al. 1975, Kiessling, Klein et al. 1975). As NK cells can be "armed and ready to kill", they need rigorous mechanisms that control their activity and prevent unregulated killing (Bryceson, Chiang et al. 2011). While writing his doctoral thesis in 1981, Klas Kärre observed that some tumor cells downregulate major histocompatibility complex (MHC) class I during progression, which led to the proposal of the "missing-self" hypothesis. In 1986, Klas Kärre and Rolf Kiessling performed experiments where they detected that tumor cells that lack MHC class I were killed by NK cells while the same cell line expressing normal levels of MHC class I was not (Karre, Ljunggren et al. 1986, Ljunggren and Karre 1990). With the identification of inhibitory receptors specific for MHC class I this hypothesis could be further proven (Yokoyama, Kehn et al. 1990, Yokoyama, Ryan et al. 1991, Colonna and Samaridis 1995, Wagtmann, Biassoni et al. 1995). Typically, NK cells are seen as a part of the innate immune system as their surface receptors trigger an inhibitory or activating signal, which determines the fate of the other cell based on cell-cell contact (Lanier 2013). It is worth mentioning that all NK cell receptors are germline-encoded and are fully functional without prior chromosomal rearrangement.

The overall balance of activating and inhibitory signals on virally infected or cancer cells will determine the outcome of NK cell reaction. In humans, inhibitory signals are mediated through recognition of self-human leukocyte antigen (HLA) class I on all cells by killer immunomodulatory-like receptors (KIRs) or other non-KIR inhibitory receptors on NK cells. According to current knowledge, during differentiation and maturation NK cells acquire one or more inhibitory receptors (NKG2A and KIRs) stochastically. So far, three inhibitory HLA class I ligand groups have been found to be crucial for NK cell function: KIR2DL1 detects HLA-C2 group antigens; KIR2DL2/DL3 is specific for HLA-C1, and KIR3DL1 detects the HLA-Bw4 epitope (Litwin, Gumperz et al. 1994, Gumperz, Litwin et al. 1995, Wagtmann, Rajagopalan et al. 1995, Pittari, Vago et al. 2017). The non-KIR inhibitory receptors like the CD94/NKG2A heterodimer engage with HLA-E, while CD161 recognizes lectin-like transcript 1 (Lanier, Chang et al. 1994, Braud, Allan et al. 1998, Lee, Llano et al. 1998, Aldemir, Prod'homme et al. 2005). The expression of at least one inhibitory signal gives NK cells the "license to kill" as there is a need for a regulatory mechanism that counteracts the activating signal.

On the other hand, NK cells also express a broad variety of different activating receptors such as natural cytotoxicity receptors (NCRs) NKp46 (CD335), NKp44 (CD336) and NKp30 (CD337), which are very potent and almost exclusively expressed on NK cells (Sivori, Vitale et al. 1997, Pessino, Sivori et al. 1998, Vitale, Bottino et al. 1998, Pende, Parolini et al. 1999). NKp46 and NKp44 are known to recognize several viral hemagglutinins, while NKp30 binds to HLA-B-associated transcript 3 (BAT3), B7-H6 and BAG6 (Arnon, Lev et al. 2001, Mandelboim, Lieberman et al. 2001, Pogge von Strandmann, Simhadri et al. 2007, Brandt, Baratin et al. 2009, Rusakiewicz, Perier et al. 2017). It is very likely that not all ligands have been discovered yet. With the exception of one, members of another activating receptor group, called signaling lymphocytic activation molecule (SLAM), are all self-ligands

and triggered by self-engagement. The exceptional member of this family is 2B4 (CD244), which interacts with CD48 on other cells. The other two members are NTB-A (natural killer, T and B cell antigen) and CRACC (CD2-like receptor activating cytotoxic cells, CD319, CS1) which both engage with themselves (Cruz-Munoz, Dong et al. 2009). Belonging to the NKG2 receptor family, NKG2D (CD314) is specific for detection of several stress-induced ligands, including the MHC-related ligands MICA (MHC class I polypeptide-related sequence A) and MICB as well as the human cytomegalovirus glycoprotein (UL16)-binding proteins ULBP1-6 (Bauer, Groh et al. 1999, Cosman, Mullberg et al. 2001). Similar to its inhibitory counterpart CD94/NKG2A, the activating heterodimer CD94/NKG2C (CD159c) binds to HLA-E (Gumperz, Litwin et al. 1995). NK cells can get activated via low affinity immunoglobulin gamma Fc region receptor III (FcγRIII, CD16). Upon activation, NK cells form a synapse with the target cell and release the lytic proteins perforin and granzyme which consequently lead to lysis of the target cells (Perussia, Acuto et al. 1983, Titus, Perez et al. 1987, Garrido, Perez et al. 1990, Mandelboim, Malik et al. 1999). Additionally, the SLAM-related surface receptor 2B4 (CD244) detects CD48; while DNAM-1 (DNAX Accessory Molecule-1) recognizes the poliovirus receptor (PVR, CD155) and Nectin-2 (CD112) (Bottino, Castriconi et al. 2003, Castriconi, Dondero et al. 2004, Fuchs, Cella et al. 2004, Tahara-Hanaoka, Shibuya et al. 2006, El-Sherbiny, Meade et al. 2007).

Hematopoietic stem cells (HSC) give rise to NK cells and the NK cell maturation is divided into five stages (Eissens, Spanholtz et al. 2012, Freud, Yu et al. 2014). To determine the five stages, the following surface markers are checked: CD34, CD117, CD94, CD56, and CD16. $CD34^{+}CD117^{-}CD94^{-}CD56^{-}CD16^{-}$identifies stage one. Stage two is defined through the acquisition of CD117 and their capability to respond to interleukin (IL)-15, which is an important feature for later NK cell development (Suzuki, Duncan et al. 1997, Carotta, Pang et al. 2011). Between stages two and three pre-NK cells lose their CD34 expression and start expressing CD56 to a small extent. Finally, mature NK cells are defined in two separate stages. Stage four is characterized as $CD56^{bright}CD94^{+}CD16^{-}$ cells while stage five NK cells express $CD56^{dim}CD94^{+/-}CD16^{+}$. The major differences between stage four and stage five NK cells are that $CD56^{dim}$ (stage five) show significantly higher cytotoxic activity and contain much more perforin and granzyme while $CD56^{bright}$ (stage four) are more efficient producers of pro-inflammatory cytokines (Cooper, Fehniger et al. 2001, Jacobs, Hintzen et al. 2001, Poli, Michel et al. 2009). It has also been shown that $CD56^{dim}$ NK cells respond better to direct receptor ligand interaction and the $CD56^{bright}$ subset responds better to soluble factors (Long, Kim et al. 2013). The ratio between $CD56^{bright}$ and $CD56^{dim}$ is about 1:9 in peripheral blood respectively. Also, the pattern of surface receptor expression differs between the $CD56^{bright}$ and $CD56^{dim}$ populations. $CD56^{dim}$ express CD16 and inhibitory KIRs while $CD56^{bright}$ are negative for CD16a and KIRs but positive for NKG2A and the IL-2 receptor α chain (IL-2R α /CD25) (Fehniger, Cooper et al. 2003, Ferlazzo, Thomas et al. 2004).

1.2.1 Natural killer cell effector mechanisms

The phenotype between the two mature NK cell subsets differ as described above, but more importantly their effector functions vary in regards to antibody-dependent cellular cytotoxicity (ADCC) and response to IL-2 stimulation. In the resting stage, $CD56^{bright}$ NK cells express less cytotoxic proteins, partially express CD16 and have CD94/NKG2A rather than KIRs as a sensor for self-tolerance. Upon stimulation with cytokines or via activating receptors, $CD56^{bright}$ are potent producers of cytokines (Wagner, Rosario et al. 2017). In contrast, $CD56^{dim}$ NK cells, which have KIR expression, are potent cytotoxic effector cells (Lanier, Le et al. 1986). This evidence suggests that $CD56^{bright}$ and $CD56^{dim}$ are distinct lymphocytes with unique tasks as innate immune cells and that all NK cells are not a homogenous population (Gonzaga, Matzinger et al. 2011). Although NK cells can kill malignant tumor cells or virus-infected cells without prior sensitization, they are also able to produce cytokines like TNF (tumor necrosis factor) and IFN-γ (interferon-γ)(Fauriat, Long et al. 2010).

Moreover, NK cells have been reported to secrete several other factors, including immunoregulatory cytokines such as IL-5, IL-10, IL-13, the growth factor GM-CSF, and the chemokines MIP-1α, MIP-1β, IL-8, and RANTES (Cuturi, Anegon et al. 1989, Smyth, Zachariae et al. 1991, Warren, Kinnear et al. 1995, Bluman, Bartynski et al. 1996, Oliva, Kinter et al. 1998, Fehniger, Shah et al. 1999, Roda, Parihar et al. 2006). Secretion of these factors has a regulatory impact on the immune system and thus builds a bridge between the innate and the adaptive immune system.

NK cells also share even more characteristics with the adaptive immune system. It has been shown that NK cells can be activated by dendritic cells (DC), be involved in autoimmune response and can recognize and respond to viral peptides (Arase, Mocarski et al. 2002, Ferlazzo and Munz 2004, Nelson, Martin et al. 2004, Fadda, Borhis et al. 2010). NK cells are also able to modulate DC functions. *In-vitro* assays have shown that NK cell can help in the DC maturation process through either killing immature DCs or direct stimulation of DCs (Ferlazzo, Semino et al. 2001). The fate of DCs is determined by the amount of MHC class I molecules on the DC surface and the expression of NK-activating receptors. Thus, mature DCs are spared from NK cell-mediated killing due to their high expression of MHC class I molecules (Ferlazzo, Tsang et al. 2002, Piccioli, Sbrana et al. 2002, Ferlazzo 2005). Furthermore, several groups have seen that after some viral infections or cancer, specific NK subpopulations stably expand and provide a long-lasting control over these diseases (Martin, Gao et al. 2002, Lopez-Botet, Angulo et al. 2004, Campillo, Martinez-Escribano et al. 2006, Martin, Qi et al. 2007, Alter, Rihn et al. 2009). All in all, NK cells do not only have innate but also adaptive features and can build up a memory-like NK cell pool.

Although it has been known for a while that NK cells are essential for control of viral infections, and that deficiencies in NK cell numbers in humans is associated with increased susceptibility to herpes virus infections, no antigen-specific memory-like NK cells could be detected (Orange 2002). The first evidence of NK cells showing a memory function was found in a mouse model in 2004 where O'Leary *et al.* reported that a specific subset of liver-

resident NK cells showed antigen-specific long-lived immunological recall responses to haptens (O'Leary, Goodarzi et al. 2006). Later in 2009 the same phenomenon was observed in murine cytomegalovirus (MCMV) infected mice (Sun, Beilke et al. 2009). The m157 glycoprotein, which is expressed on MCMV infected cells, can induce a selective expansion and activation of NK cell subsets that are long lasting and show memory-like features (Dokun, Kim et al. 2001). Upon re-challenging with MCMV those MCMV-specific NK cells can respond more rapidly and effectively than naive NK cells (Sun, Beilke et al. 2009). Similar to MCMV, human CMV (HCMV) is also able to induce expansion of a specific subpopulation that is defined as CD94/NKG2C^{+}CD57^{+} which is highly specific to HCMV, although the ligand for this has not yet been identified (Guma, Angulo et al. 2004, Lopez-Verges, Milush et al. 2011, Della Chiesa, Falco et al. 2013, Hendricks, Balfour et al. 2014, Muntasell, Pupuleku et al. 2016).

Upon engagement with a target cell and in case the activating signal is more dominant than the inhibitory signal, polarization and exocytosis of granules towards the target cells are initiated. The released granules are filled with perforin and granzyme. The role of perforin is to create pores in the target cell membrane, while that of granzyme is to enter the cell through these pores to then induce caspase-mediated apoptosis (Bryceson, March et al. 2006, Voskoboinik, Smyth et al. 2006). In addition to perforin and granzyme mediated apoptosis after degranulation, NK cells are also able to kill by receptor-ligand interaction. Tumor necrosis factor ligand superfamily member 6 (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) are known as death ligands on NK cells. Their corresponding receptors Fas and TRAILR found on tumor cells induce apoptosis in the target cells (Medvedev, Johnsen et al. 1997). The tumor microenvironment can downregulate activating ligands and can skew the NK cell phenotype due to secretion of cytokines to escape immune surveillance (Jinushi, Takehara et al. 2005, Konjevic, Mirjacic Martinovic et al. 2007). Therefore, it is critical for NK cells to have an alternative mechanism of targeting tumor cells in cases where the ligand is shed or activating receptors are downregulated (Lundqvist, Abrams et al. 2006).

1.2.2 Natural killer cells in multiple myeloma

As previously stated, the control of NK cell-mediated tolerance needs a delicate balance between activating and inhibiting signals that regulate the activity of NK cells in the steady state. When a tumor progresses, this equilibrium is usually out of balance due to the influence of tumor cells on the homeostasis of the immune system. Detection of MHC class I on the surface of tumor cells can inhibit NK cell killing, while the lack of MHC class I diminishes the inhibitory signal, which can lead to lysis of the tumor cell.

Different tumors have found distinct ways to overcome NK cell-mediated killing. Some tumor cells are able to induce apoptosis in NK cells by engaging with NCRs. Engagement of those receptors leads to upregulation of FasL mRNA and consequently protein synthesis, which can interact with Fas at NK cell surface and induce suicide (Poggi, Massaro et al. 2005). Costello *et al.* show that in acute myeloid leukemia (AML), NCRs on the cell surface

are significantly down-regulated (Costello, Sivori et al. 2002). Additionally, numerous other malignancies show a modulation of the immune system by the tumor to escape detection or killing (Zitvogel, Tesniere et al. 2006). Hepatocellular carcinoma, metastatic melanoma, chronic lymphocytic leukemia (CLL), and multiple myeloma regulate the NK cell population in a way that the detection of the tumor is defective (Jinushi, Takehara et al. 2005, Fauriat, Mallet et al. 2006, El-Sherbiny, Meade et al. 2007, Konjevic, Mirjacic Martinovic et al. 2007, Veuillen, Aurran-Schleinitz et al. 2012). This can happen through upregulation of MHC class I on the tumor cells or by modulation of the NK cell phenotype and function (Pierson and Miller 1996, Classen, Falk et al. 2003, Costello, Fauriat et al. 2004).

Additionally, tumor cells can also escape immune surveillance by downregulation of activating ligands or through ligand shedding. MICA and MICB, which are the natural ligands for the activating receptor NKG2D, are overexpressed in malignant transformed cells, due to cellular stress (Pende, Rivera et al. 2002). However, to avoid NK immune surveillance, MM cells and other tumors can shed membrane-bound MIC (Jinushi, Takehara et al. 2005, Boissel, Rea et al. 2006, Jinushi, Vanneman et al. 2008, Kohga, Takehara et al. 2008). The presence of soluble MIC (sMIC) leads to internalization of surface NKG2D as well as NCRs which consequently impair NK cell effectiveness (Groh, Wu et al. 2002, Doubrovina, Doubrovin et al. 2003, Wu, Higgins et al. 2004). In line with these findings it is not surprising that the presence of sMIC is associated with poor cancer survival (Pittari, Vago et al. 2017). The same mechanism applies to the NCR NKp30 where circulating BAT3 can inhibit NK cell cytotoxicity and also leads to NKp30-specific hypo-responsiveness (Reiners, Topolar et al. 2013).

Cancer cells are not only able to shed surface ligands to avoid detection. They also frequently upregulate non-classical MHC class I molecule HLA-G, which dampens NK cell response by activating the inhibitory receptors ILT-2 and KIR2DL4 (Rouas-Freiss, Moreau et al. 2005, Urosevic and Dummer 2008). Additionally, the upregulation of the non-classical HLA class I antigen HLA-E is related to a poor prognosis, and it could be shown that MM cells with increased HLA-E expression are less susceptible to killing by $NKG2A^+$ NK cells (Bossard, Bezieau et al. 2012, Sarkar, van Gelder et al. 2015). Cell lines that were created from MM patients showed downregulated B7-H6 resulting in an NKp30-mediated impairment of NK cell functionality (Fiegler, Textor et al. 2013). Interestingly, early stage MM patients show low levels of HLA class I expression, while plasma cells from patients with advanced MM show high HLA class I expression which could potentially be a mechanism to avoid NK cell-mediated killing due to induction of inhibitory signaling pathways on NK cells (Carbone, Neri et al. 2005).

Modulation of the BM and creation of an immunosuppressive milieu is not the only effect that the progression of MM has on the NK cell population. It has been found that in early stage and/or untreated MM, NK cell counts are similar or sometimes even elevated (Omede, Boccadoro et al. 1990, Osterborg, Nilsson et al. 1990, Famularo, D'Ambrosio et al. 1992). This would suggest that NK cell-mediated surveillance controls the disease, but it could also be understood as an effect of immunological stress due to poor disease management (Pittari, Vago et al. 2017). NK cells from MM patients also have an exhausted phenotype that includes

downregulation of several activating receptors and upregulation of programmed death receptor (PD)-1. The activating receptors that are affected by the downregulation are 2B4, NKG2D and also the NCRs, which are both decreased in circulating and in BM NK cells (Fauriat, Mallet et al. 2006, Costello, Boehrer et al. 2013). Compared to healthy donors or MM patients in remission, expression levels of DNAM-1 are also lower in patients with active MM, which consequently has an impact on late-stage tumor immune escape due to lack of interaction with PVR and nectin-2 that are most important for cancer cell elimination (El-Sherbiny, Meade et al. 2007, Guillerey, Ferrari de Andrade et al. 2015). Therefore, the phenotype and function of NK cells in active MM is skewed and hence the immune surveillance by NK cells is disturbed, which leads to immune escape of MM (Jurisic, Srdic et al. 2007).

1.3 BONE MARROW MICROENVIRONMENT IN MULTIPLE MYELOMA

MM develops in the BM, which is the primary site of hematopoiesis in adults. Hematopoietic stem cells give rise to several types of blood cells like immune cells, erythrocytes, platelets and others. Moreover, the microenvironment in the BM provides optimal conditions for maintenance, proliferation, and differentiation of various cell types and provides the primary home of plasma cells (Wilson and Trumpp 2006, Tangye 2011, Chu and Berek 2013). The BM microenvironment contains cellular compartments, extracellular matrix and also soluble factors like cytokines, chemokines and growth factors which are needed to form the BM niche (Romano, Conticello et al. 2014, Kawano, Moschetta et al. 2015).

In the early stages of MM, it has been shown that the cells are strictly dependent on the BM and even in later stages the dependency on a tumor supporting environment is crucial for MM cell proliferation (Kuehl and Bergsagel 2002); additionally *ex-vivo* studies revealed that culturing MM cells is very challenging (Hughes 2011, Duru, Sutlu et al. 2015). Probably the most essential factor for MM cell growth in the BM is the cytokine IL-6 (Hirano and Kishimoto 1989, Suematsu, Hibi et al. 1990). Interestingly, the response to IL-6 stimulation is quite different in normal and malignant plasma cells. While IL-6 stimulates the production of Ig in normal plasma cells, it promotes proliferation and resistance to apoptosis in MM cells (Mitsiades, McMillin et al. 2007). Although MM cells can produce IL-6, the predominant source is other cell types like T cells, B cells and bone marrow stromal cells (BMSC) (Kishimoto, Hibi et al. 1992, Gunn, Conley et al. 2006). Together with the high levels of IL-6, IL-10 is also increased in MM and acts as a growth factor for plasma cells (Bataille, Jourdan et al. 1989, Zhang, Klein et al. 1989, Kovacs 2010, Sharma, Khan et al. 2010, Zheng, Zhang et al. 2013). The effects of both IL-6 and IL-10 on the immune system impair NK cell activity and inhibit the production of IFN-γ and TNF, which are pro-inflammatory cytokines (Tsuruma, Yagihashi et al. 1999, Conti, Kempuraj et al. 2003, Cifaldi, Prencipe et al. 2015). The MM cells together with regulatory T cells (T_{reg}) create an immunosuppressive milieu due to the production of high levels of transforming growth factor (TGF)-β, which is known to downregulate the expression of activating receptors. This affects not only NK cell cytotoxicity but also leads to impaired T cell response and defective antigen presentation by DCs (Urashima, Ogata et al. 1996, Cook and Campbell 1999, Castriconi, Cantoni et al.

2003, Lee, Lee et al. 2004, Pinzon-Charry, Maxwell et al. 2005, Beyer, Kochanek et al. 2006, El-Sherbiny, Meade et al. 2007).

Cytokines modulate the immune response in the BM, but also other soluble factors are known to suppress NK cell functions. Prostaglandin E2 inhibits signal transduction from several activating receptors and Indoleamine 2,3-dioxygenase converts the essential amino acid l-tryptophan into l-kynurenine and thus inhibits immune effector cells by depletion of l-tryptophan (Lu, Bataille et al. 1995, Mellor and Munn 1999, Munn, Shafizadeh et al. 1999, Martinet, Jean et al. 2010).

One of the hallmarks of cancer is inflammation and it has been well described that an inflammatory microenvironment promotes tumor growth (Colotta, Allavena et al. 2009, Grivennikov, Greten et al. 2010). In solid tumors myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM) play a pivotal role in dampening the body's immune response and creating a tumor friendly milieu (Berardi, Ria et al. 2013). This has not been proven for hematological malignancies, but there is emerging evidence that the same cell types are involved (Berardi, Ria et al. 2013). MDSC levels are elevated in MM and they can directly contribute to reduced NK cell function through membrane bound tumor growth factor (TGF-β) and TIGIT (T cell immunoreceptor with Ig and ITIM domains)-mediated inhibitory signaling towards the DNAM-1 signaling pathway (Li, Han et al. 2009, Van Valckenborgh, Schouppe et al. 2012, Zhuang, Zhang et al. 2012, Sarhan, Cichocki et al. 2016).

MM cells do not only change the BM niche into a milieu that accommodates their needs and promotes proliferation, but they also change the immune response of the body. Altogether, there is emerging evidence that the change in the immune system is driving MGUS to MM progression and it thus has an essential role in disease progression (Dhodapkar, Krasovsky et al. 2003, Perez-Andres, Almeida et al. 2005, Bernal, Garrido et al. 2009).

1.4 HISTORY OF MULTIPLE MYELOMA TREATMENT

Historically, MM was treated with different kinds of natural medicine e.g. rhubarb pills, orange peel or urethane (Solly 1844, Macintyre 1850, Longsworth, Shedlovsky et al. 1939, Alwall 1947, Alwall 1952). In 1958, Blokhin *et al.* reported for the first time on the treatment of MM with melphalan which showed some impact on lowering serum levels, although a survival benefit could not be observed (Blokhin, Larionov et al. 1958, Bergsagel, Sprague et al. 1962, Mass 1962, Hoogstraten, Sheehe et al. 1967). In 1969, Alexanian *et al.* introduced the combination of melphalan and prednisone (MP) which was the first effective therapy against MM and became the standard until the late 1990s (Alexanian, Haut et al. 1969).

MM responds to classical cytotoxic, immunomodulatory and other targeted drugs and also to cell-based therapies like autologous and allogeneic stem cell transplantation (Gahrton 2004). The overall survival (OS) of patients with newly diagnosed MM has increased from approximately three years during the years 1985–1998 to six to ten years today (Kyle, Gertz et al. 2003, Moreau, Attal et al. 2015). Due to the persistence of residual tumor cells, MM

is still considered to be an incurable disease and all patients eventually relapse. However, new and improved therapies or combinations have considerably improved survival rates and quality of life for many patients (Kaufmann, Urbauer et al. 2001, Alici, Bjorkstrand et al. 2007).

1.4.1 Immunomodulatory drugs

The treatment possibilities have progressed dramatically with the introduction of the first novel agent, Thalidomide, which was the first immunomodulatory drug (IMiD) used in treating MM in 1999 (Singhal, Mehta et al. 1999). Initially, Thalidomide was developed in West Germany in the 1950s and sold as a sedative and a treatment modality of pregnancy-related morning sickness and nausea. The drug was withdrawn from the market in the beginning of the 1960s due to its severe teratogenic side effect (Kyle, Gertz et al. 2003, Badros, Goloubeva et al. 2005). As the name IMiD implies, this drug type not only inhibits the proliferation of the malignant cells and hampers the interaction of tumor cells and their microenvironment, but also interact with the immune system by activation of T cells and NK cells (Quach, Ritchie et al. 2010).

Although Thalidomide in combination with MP has shown an OS benefit of six months, researchers and clinicians have been searching for drugs with fewer side effects and higher potency (Fayers, Palumbo et al. 2011). In line with this, Lenalidomide (Len) was approved in 2006 and consequently Pomalidomide (Pom) in 2012, which are now considered second generation IMiDs.

1.4.2 IMiDs and mechanism of action

The mechanisms of action for IMiDs are multifarious. A critical part of the immune modulation is the binding of Len to the protein cereblon (Ito, Ando et al. 2010). IMiDs target two ubiquitin ligase complexes directly, which consequently leads to the degradation of the transcription factors IKZF1 and IKZF3 (Ikaros and Aiolos, respectively) (Kronke, Udeshi et al. 2014). Followed by a cascade reaction, those missing transcription factors lead to down-regulation of the transcription factors IRF4 and MYC which are essential for MM cell survival (Shaffer, Emre et al. 2008).

The survival of MM cells is facilitated by an impairment of the immune system (Zou 2005). MM skews the immune system in a way that B and T cells are inhibited by myeloma-derived cytokines like TGF-β as well as inadequate antigen presentation, resistance to NK cell lysis, and defective T, B and NK cells (Brown, Pope et al. 2001, Smyth, Godfrey et al. 2001, Brimnes, Svane et al. 2006). Additionally, both humoral and cellular immunity are damaged. Thus, MM is associated with impaired B-cell differentiation and antibody responses, reduced T cell numbers, more specifically $CD4^+$ T cells, abnormal T helper (Th)1/Th2 $CD4^+$ T cell ratio, impaired cytotoxic T cell responses, dysfunction of NK and NKT cells, and defective DC function (Rawstron, Davies et al. 1998, Brown, Pope et al. 2001, Dhodapkar, Geller et

al. 2003, Maecker, Anderson et al. 2003, Ogawara, Handa et al. 2005, Jarahian, Watzl et al. 2007).

Interaction of Thalidomide or other IMiDs with the immune system breaks the myeloma cell "tolerance" by co-stimulation of T cells, interaction with T_{reg}s and enhancement of NK and NKT cells (Quach, Ritchie et al. 2010). Both $CD4^+$ and $CD8^+$ T cells will be skewed towards enhanced production of Th1 type cytokines and show enhanced proliferation (Davies, Raje et al. 2001). In terms of co-stimulation and induction of proliferation, Len is more potent than Thalidomide (Corral and Kaplan 1999, Davies, Raje et al. 2001). Pom appears to induce co-stimulation even better than Len and shows similar Th1 type cytokine production (Schafer, Gandhi et al. 2003). The exact mechanisms and targets by which T cell proliferation and activation are induced by IMIDs are currently unknown. However, it has been shown that both Thalidomide and Pom enhance the activity of activator protein-1 (AP-1), which is a key driver to IL-2 production (Schafer, Gandhi et al. 2003). The increased production of IL-2 promotes NK cell proliferation and activation and thus has a direct effect on the activity of the innate and adaptive immune system (Davies, Raje et al. 2001, Hayashi, Hideshima et al. 2005).

The previously mentioned boosting of the innate immune system by IMiDs is mainly due to NK and NKT cell activation and is well documented (Davies, Raje et al. 2001, Hayashi, Hideshima et al. 2005). The increased production of IL-2 by activated T cells leads to a direct stimulation and increased function of NK cells. It has been shown by Davies *et al.* that Len, Thalidomide and Pom promote NK cell proliferation and enhanced death of MM cell lines and also primary patient cancer cells *in-vitro* (Davies, Raje et al. 2001). Interestingly, only Pom and Len can induce enhanced ADCC and natural cytotoxicity of NK cells in addition to their increase in proliferation (Hayashi, Hideshima et al. 2005, Tai, Li et al. 2005). It is also interesting to note that the *in-vitro* augmentation of ADCC on NK cells by IMiDs requires both antibody (Ab) binding to Fc-γ receptors on NK cells, as well as the presence of IL-2 (Hayashi, Hideshima et al. 2005, Tai, Li et al. 2005). So far, *in-vitro* studies have demonstrated that IMiDs stimulate T cell and NKT cell production of IL-2 and IFN-γ, which has a direct impact on NK cell-mediated cytotoxicity and proliferation. NK cells in turn produce cytokines like monocyte-recruiting chemotactic protein (MCP-1) and GM-CSF in response to Ab-coated target cells, which recruit DCs and T cells (Roda, Parihar et al. 2006). This results in further chemotactic attraction of tumor-specific T cells in the presence of IMiDs. A summary over the various effects of IMiDs on the immune system is shown in Figure 1.

All in all, the development of IMiDs as a single agent or combination therapy together with autologous stem cell transplantation (ASCT) has drastically improved the treatment possibilities. Although for the majority of patients the standard treatment is still different chemotherapeutical agents that target and destroy the cancer cells. New combination therapies with proteasome inhibitors (PIs) and IMiDs lead to higher response rates compared to chemotherapy alone and thus to improved OS (Dimopoulos, Zervas et al. 2001, Mitsiades, Hideshima et al. 2009, Ponisch, Andrea et al. 2012, Mina, Cerrato et al. 2016). Particularly the combination with bisphosphonates leads to response rates of up to

80% in newly diagnosed patients and up to 75 % in patients with a relapsed disease (Garcia-Sanz, Gonzalez-Fraile et al. 2002, Palumbo, Bertola et al. 2005). In addition to the conventional therapeutic approaches, the highest hopes for curing this disease rest on immunotherapy. Utilizing the body's own immune system with monoclonal antibodies (mAbs), activated or genetically modified cells have the characteristics to target the tumor more precisely and direct while improving cytotoxicity with lower collateral damage to other cells and tissue of the patient.

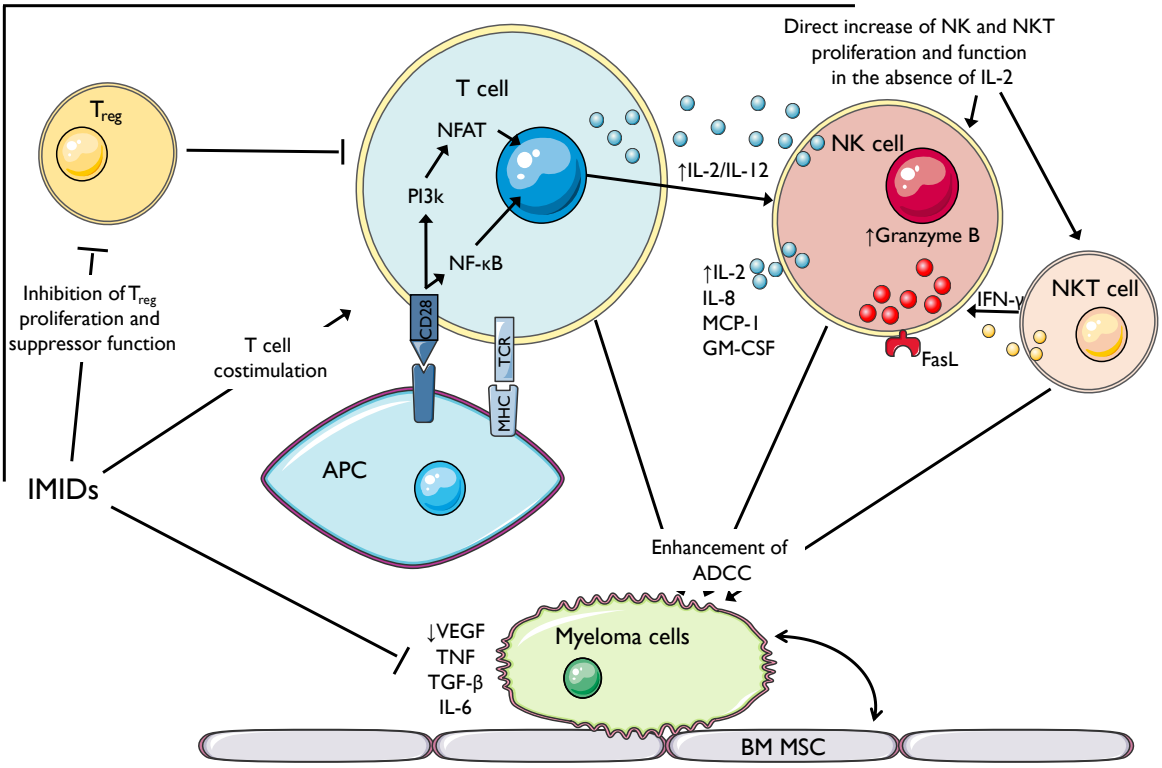

Figure 1: Overview of the immunomodulatory outcomes of immunomodulatory drugs. BMSC: bone marrow stromal cells; APC: antigen-presenting cells; IL: interleukin; TGF: transforming growth factor; TNF: tumor necrosis factor; VEGF: vascular endothelia growth factor; ADCC: antibody-dependent cellular toxicity; MHC: major histocompatibility complex; TCR: T cell receptor; NF-κB: Nuclear factor kappa B; PI3k: phosphoinositide 3-kinase; NFAT: nuclear factor of activated T cell; IFN: interferon; NK: natural killer.
Adapted from (Quach, Ritchie et al. 2010)

1.4.3 Proteasome inhibitors in multiple myeloma

The ubiquitin-proteasome pathway (UPP) which is the major pathway for intracellular protein degradation can be defective in cancer cells. Thus, inhibiting this pathway would prevent malignant cells from proliferating (Voorhees, Dees et al. 2003, Crawford, Walker et al. 2011). Blocking the UPP with PIs is particularly useful in MM cells. Upon treatment with PIs, misfolded Ig accumulate in the endoplasmatic reticulum (ER) which then activates the proteasome function (Obeng, Carlson et al. 2006). This creates prolonged stress in the MM cells which consequently activates pathways that lead to cell cycle arrest and induction of apoptosis (McCullough, Martindale et al. 2001).

Currently, three different PIs are approved for the treatment of MM. The oldest one, Bortezomib (Bort), was approved in 2003 after it showed a good response rate in relapsed/refractory MM (RRMM) as a single agent treatment and also in combination with IMiDs or alkylating agents like Dexamethasone (Dex) (Chauhan, Catley et al. 2005, Richardson, Sonneveld et al. 2005, Richardson, Xie et al. 2009). The second one, Carfilzomib (Carf), was approved in the US in 2012 and in Europe in 2016. Its chemical moiety is different from Bort, and the binding effect is irreversible, which means that restoration of proteasome activity is only possible by new synthesis of the required subunits (Kuhn, Chen et al. 2007). Similar to Bort, Carf has shown to be effective as a single agent drug, but the most effective treatment is also as a combination therapy with Len as demonstrated in the ASPIRE study (Papadopoulos, Siegel et al. 2015, Stewart, Rajkumar et al. 2015). The third approved PI is Ixazomib which was approved in the US in 2015 and can be administered orally. The chemical moiety is similar to Bort and targets the same subunit of the proteasome with greater potential activity against MM cells (Chauhan, Tian et al. 2011). In addition to those three approved PIs, several more are currently being tested in numerous clinical trials which should provide a better clinical outcome and reduced toxicity for MM patients (Kubiczkova, Pour et al. 2014).

1.5 IMMUNOTHERAPY IN MULTIPLE MYELOMA

Immunotherapy is defined as a treatment that uses certain parts of a person's immune system to fight diseases such as cancer by either stimulating the body's own immune system or giving the immune system supporting components that are man-made and have direct influence on the disease.

Immunotherapy has proven to be a highly active area in cancer therapeutics that is being explored in patients with myeloma. Strategies stretch from currently used treatments to experimental approaches that are investigated in various clinical trials. Those strategies include mAbs to target myeloma-associated antigens, checkpoint inhibitors to induce T-cell activation, engineered effector cells to target myeloma cells, and vaccine therapy to restore tumor-specific T cells within the immune effector repertoire (Boussi and Niesvizky 2017).

The most common form of immunotherapy is allogeneic stem cell transplantation (allo-SCT) where the donor's immune system is utilized to target the cancer cells. Allo-SCT has been shown to yield a durable response in MM patients when receiving grafts from HLA-matched sibling donors and is the standard treatment in eligible MM patients. (Gahrton, Tura et al. 1991, Martino, Recchia et al. 2016). ASCT can be used as either single or double treatment, if necessary. The use of allo-SCT following ASCT or as a salvage therapy on relapse is currently not recommended outside clinical trials or very specific patient conditions (Martino, Recchia et al. 2016). Several studies that compared double ASCTs with ASCT followed by reduced-intensity conditioning allo-SCT have yielded mixed results without any consistent OS benefit for the patients (Garban, Attal et al. 2006, Bruno, Rotta et al. 2007, Bjorkstrand, Iacobelli et al. 2011, Martino, Recchia et al. 2016). But it is worth mentioning that those studies have been overshadowed by treatment-related morbidity and mortality and that some patients did benefit from allo-SCT treatment.

1.5.1 Monoclonal antibodies in the treatment of multiple myeloma

Monoclonal antibodies are the newest approach in the treatment of hematological malignancies. The first monoclonal antibodies approved by the US Food and Drug Administration (FDA) were for the treatment of B cell non-Hodgkin lymphoma (NHL), and they revolutionized the treatment outcome. The introduction of rituximab, an anti-CD20 mAb, has improved the OS compared to the standard treatment by 10-15% in all age groups and showed no high toxicity (Coiffier, Lepage et al. 2002, Sehn, Donaldson et al. 2005). This success story has encouraged researchers to develop mABs for the treatment of MM as well. So far, two mABs have obtained FDA and European Medicines Agency (EMA) approval for treatment of MM: Daratumumab and Elotuzumab (Table I).

Table 1: Daratumumab and Elotuzumab: targets, mechanisms of action and approved indications.

Name	Target	Mechanism of action	Completed Studies (Phase)	Ongoing studies (Phase)	Indication
Elotuzumab	CS1/SLAMF 7/CRACC	ADCC, direct activation of NK cells	RRMM (I/II/III)	Newly diagnosed (III) SMM (II)	Combination with Len-Dex (1-3 prior tx)
Daratumumab	CD38	ADCC, ADCP, CDC, apoptosis via cross-linking, depletion of T_{reg}s	RRMM (I/II/III)	Newly diagnosed (III) SMM (II)	Combination with Len-Dex or Bort-Dex (> 1 prior tx); monotherapy after at least 3 prior tx

ADCC, antibody-dependent cell-mediated cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; Bort, bortezomib; CDC, complement- dependent cytotoxicity; Dex, dexamethasone; Len, lenalidomide; RRMM, relapsed/refractory multiple myeloma; SMM, smoldering multiple myeloma; tx, treatment.

1.5.2 Targeting CD319 on multiple myeloma cells

Elotuzumab (Elo) is a humanized mAb which is used in MM treatment. It specifically targets CS1 (also called SLAMF7,CD319 or CRACC), which is a surface marker and member of the signaling lymphocyte activation molecule-related receptor family (SLAMF) (Kumaresan, Lai et al. 2002). CS1 is highly expressed on plasma cells in patients suffering from MGUS or MM and keeps high expression levels regardless of previous lines of therapy. However, other cell types like NK or NKT cells, as well as some T cell subsets and activated monocytes also express CSI although expression levels are significantly lower. No expression of CS1 could be found in healthy tissue, which allows a targeted treatment with minimal side effects (Hsi, Steinle et al. 2008, Tai, Dillon et al. 2008).

The primary mechanism of action of Elo is binding directly to CS1 on the MM cells and inducing NK cell-mediated ADCC which is summarized in Figure 2 (Varga, Maglio et al. 2018) This mechanism of action has been proven in several *in-vitro* studies and it could also be shown that NK cells could induce ADCC in MM cells from patients resistant to previous IMiDs or PIs treatments (Tai, Dillon et al. 2008). Initially tested in RRMM patients during a phase I study, Elo indicated to be a well-tolerated drug with only modest activity as monotherapy (Zonder, Mohrbacher et al. 2012). In later studies, the combination with Len or Bort led to an overall response rate which was more impressive and additionally

increased the median time to progression (Jakubowiak, Benson et al. 2012, Lonial, Vij et al. 2012).

Later during a phase III study, Lonial et al. compared the combination of Elo with Len and Dex (ELd) against Len and Dex (Ld) alone. Progression-free survival (PFS) in the ELd group was significantly higher compared to the Ld group. Also, treatment-related side effects were lower in ELd treated patients. Interestingly, the infection rates were comparable except for the infection rate of herpes zoster, which was higher in the Elo group (Lonial, Dimopoulos et al. 2015). Overall it could be shown that the addition of Elo to Len and Dex led to a reduced risk of disease progression by 30%. Based on this study the FDA approved the use of Elo as a treatment option for patients with MM who had received one to three prior therapies (Varga, Maglio et al. 2018).

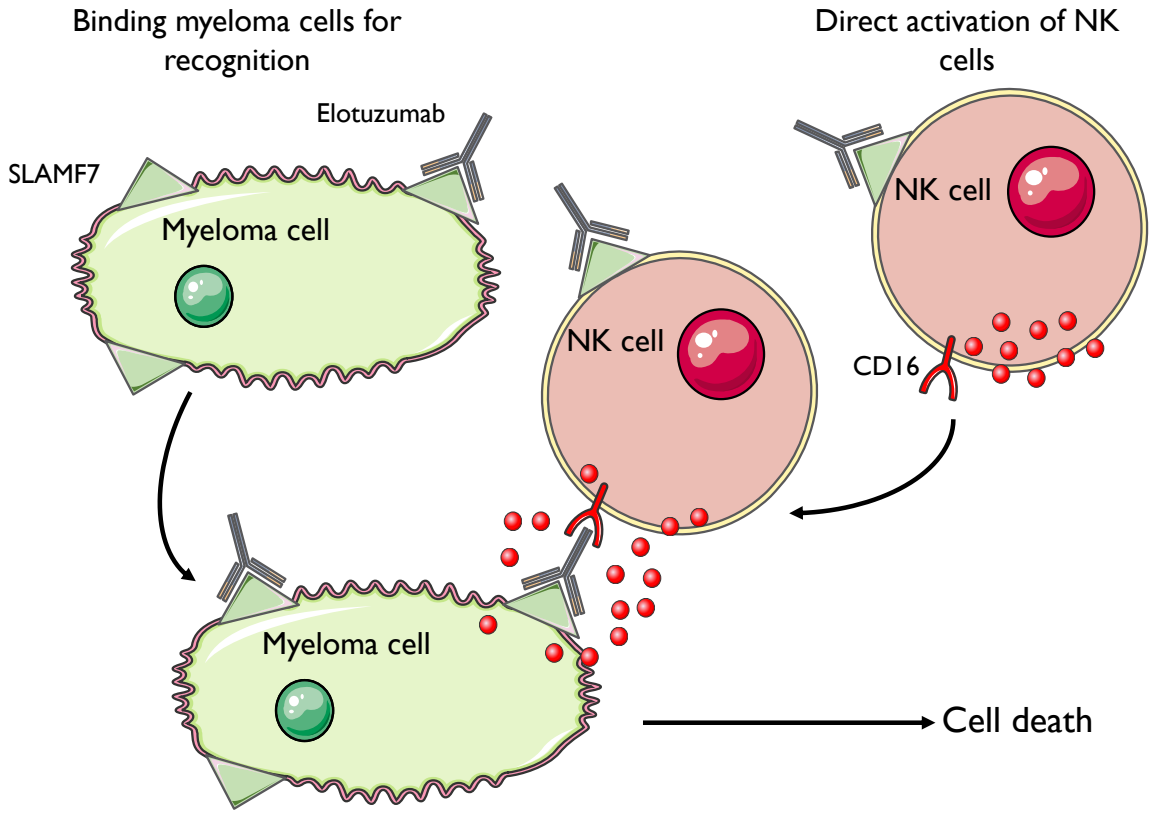

Figure 2: Mechanism of action for Elotuzumab. CD: cluster of differentiation; SLAMF7: signaling lymphocytic activation molecule family member 7 (CS1, CD319); NK: natural killer. Adapted from (Varga, Maglio et al. 2018)

1.5.3 Targeting CD38 on multiple myeloma cells

CD38 is a 45 kDa type II transmembrane glycoprotein, which is highly and uniformly expressed on myeloma cells and plays a role in receptor-mediated signaling events for cell adhesion (Lin, Owens et al. 2004, Santonocito, Consoli et al. 2004, Deaglio, Vaisitti et al. 2007); additionally, CD38 also plays a pivotal role in the intracellular mobilization of calcium

(Deshpande, White et al. 2005). CD38 is expressed at low levels on normal lymphoid and myeloid cells but shows very high expression on malignant plasma cells in all stages of MM, making CD38 a valuable target for antibody therapy (Lin, Owens et al. 2004, Malavasi, Deaglio et al. 2008, Malavasi 2011). Although several anti-CD38 antibodies have been investigated, only Daratumumab (Dara), which has a fully humanized IgG1-κ isotype, received FDA-approval. Three other antibodies, Isatuximab, MOR202 and TAK-079 are currently tested in phase III and phase I/II studies, respectively (Martin, Hsu et al. 2014, Martin, Mannis et al. 2016, Richter, Martin et al. 2016, van de Donk, Richardson et al. 2018). Notably, Isatuximab received orphan drug status in the beginning of 2019 when a phase III clinical trial met its primary endpoint of improved PFS in combination with Pom plus Dex compared to Pom plus Dex alone in RRMM.

Dara specifically targets a unique epitope on CD38 and can destroy MM cells through multiple direct and indirect mechanisms (van de Donk, Moreau et al. 2016, Varga, Maglio et al. 2018). Coating of the target MM cells by Dara leads to detection and activation of effector cells that will kill the MM cells via ADCC through the release of cytotoxic granules or binding of cell death-inducing molecules like FasL (de Weers, Tai et al. 2011). Another mechanism of action is via complement dependent cytotoxicity (CDC), which starts by binding of the antibody and activation of the complement cascade, leading ultimately to cell lysis and death due to the interaction of the complement system with the cell membrane (de Weers, Tai et al. 2011). The third indirect killing mechanism is antibody-dependent cellular phagocytosis (ADCP), which is mediated by macrophages (Overdijk, Verploegen et al. 2015). Recently, it was also shown that Dara has a direct effect on the numbers of Tregs, which express high levels of CD38 and can therefore be a depleted due to Dara-mediated ADCC (Krejcik, Casneuf et al. 2016). An overview of the different effects of Dara is shown in Figure 3.

Lenalidomide can be a potent inducer of NK cell activity and proliferation and it was previously shown that Len in combination with an anti-CD20 mAb, Rituximab, increases the ADCC effect in chronic lymphocytic leukemia (CLL) (Byrd, Peterson et al. 2005, Friedberg 2008). This discovery led to the assumption that the combination of Dara with Len could also show synergistic effects. These were explored by Veer *et al.* in pre-clinical studies which demonstrated promising first results (van der Veer, de Weers et al. 2011). The POLLUX study later confirmed that combining Dara with Len induces a deep response in RRMM patients that lasted for over two years with a very favorable safety profile (Dimopoulos, Oriol et al. 2016, Moreau, Oriol et al. 2017, Plesner, Arkenau et al. 2017, Dimopoulos, San-Miguel et al. 2018).

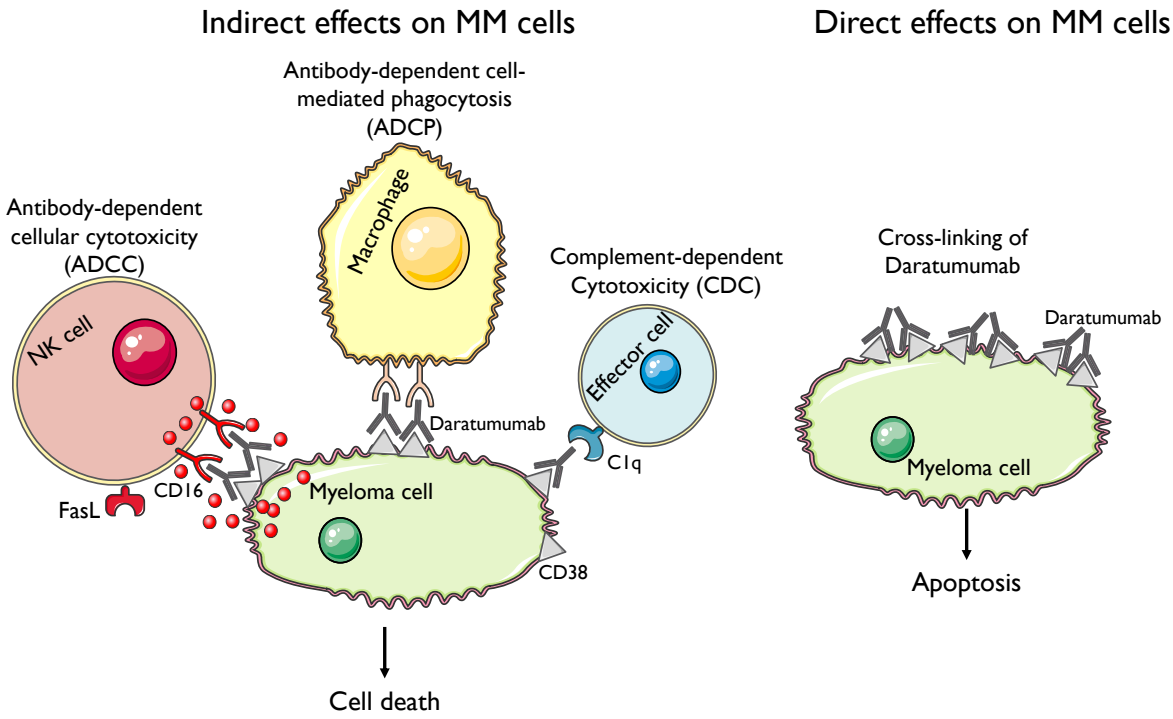

Figure 3: Mechanism of action for Daratumumab. CD: cluster of differentiation; C1q: complement component 1q; FasL: Tumor necrosis factor ligand superfamily member 6; NK: natural killer.
Adapted from (Varga, Maglio et al. 2018)

1.5.4 Co-inhibitory molecules

The costimulatory pathway that is controlled by PD1/PD-ligand (PD-LI) helps maintain T cell homeostasis and also protects against autoimmunity. While PD1 is expressed on the surface of T cells, B cells and NK cells the corresponding ligands PD-L1 and PD-L2 are primarily expressed on DCs and macrophages (Keir, Butte et al. 2008). Upon binding of PD-L1, T cells secrete less Th1-cytokines, proliferate less, and show lower T cell-mediated killing (Rosenblatt and Avigan 2017). MM cells, as many other cancer cells, abuse this system and create an immunosuppressive milieu by upregulating PD-L1 expression (Liu, Hamrouni et al. 2007, Gajewski, Schreiber et al. 2013, Tamura, Ishibashi et al. 2013). PD1 is also upregulated on T cells from MM patients; thus targeting PD1/PD-L1 by immune checkpoint inhibitors is a logical consequence. Although checkpoint inhibition showed remarkable therapeutic success in some solid malignancies, this effect could not yet be confirmed in MM treatment (Gay, D'Agostino et al. 2017, Jelinek, Mihalyova et al. 2017). PD1 blockade with Nivolumab, as monotherapy, has shown discouraging results in RRMM patients in a phase 1b study, since no objective response could be observed in 67% of the patients (Lesokhin, Ansell et al. 2016). Another PD1 blocking mAb Pembrolizumab in combination with Len or Pom plus Dex showed a very good overall response rate and durable improvement in patients which are refractory to both IMiDs and PIs, although increased autoimmune disorders were observed (San Miguel, Mateos et al. 2015, Badros, Hyjek et al. 2017). Two phase III clinical trials were set on hold by the FDA in 2017, because an unexplained

increased risk for death was observed with Pembrolizumab therapy. Later in 2017, the FDA also stopped three trials exploring Nivolumab-based treatment, which could be restarted after amendments.

1.5.5 Adoptive T cell therapy

Adoptive T cell therapy is a type of immunotherapy where isolated and/or expanded T cells are given to a patient to fight a disease. The expansion and activation of bone-marrow infiltrating lymphocytes (MILs) demonstrate enhanced antitumor specificity, effectively targeting plasma cells and their clonogenic precursors. In a phase 1 trial MILs were used to treat RRMM patients in combination with ASCT and the patients showed increased PFS by thirteen months but no increased OS (Noonan, Huff et al. 2015).

One of the most promising new approaches is the use of chimeric antigen receptors (CAR) to modify T cells. The CARs consist of a variable antibody chain that specifically targets a surface molecule in their native conformation. In contrast to a T cell receptor (TCR), the CAR binds the target in an MHC-independent fashion. Initially, the first generations of CARs were engineered from a single-chain variable fragment from an antibody combined with an intracellular CD3ζ signaling domain. This construct showed poor clinical activity and persistence was very modest. To increase the effect of the CAR signaling domains, co-stimulatory molecules such as CD28, CD137 (4-1BB) or CD134 (OX40) have been added to the construct. These modifications provided additional effector functions such as cytokine production or proliferation. The combination of two of those co-stimulatory domains built the basis for the third-generation of CARs; the typical combinations are CD28 plus 4-1BB or CD28 plus OX40 (Maus, Grupp et al. 2014).The effect of CAR T cells on CD19 expressing and other B cell malignant tumor cells led to the development of CAR T cells for other targets (Maude, Frey et al. 2014, Kochenderfer, Dudley et al. 2015).

Typical targets for MM cells are B or plasma cell-related surface receptors like B cell maturation antigen (BCMA), CS1, CD38, CD138. Both CS1 and CD38 are currently used for immunotherapy utilizing mAb in combination with IMiDs or PIs. BCMA, which is a protein that regulates B cell maturation and differentiation into plasma cells, is currently one of the main targets because of its almost unique expression on plasmablasts and plasma cells (Tai, Li et al. 2006, Jung, Lee et al. 2017). In an early phase, dose-escalating trial patients received CAR-BCMA T cells and the MM specific response was promising; the four given doses were 0.3 x10^6, 1x10^6 CAR, 3 x10^6 and 9 x10^6 CAR T cells/kg body weight, respectively. Overall, out of the twelve treated patients, six patients showed stable disease (SD), one achieved partial response (PR), two had very good partial responses (VGPR), and one showed complete response (CR), which demonstrated the potent capacity of CAR-BCMA T cells in MM patients (Ali, Shi et al. 2016). Other phase 1 and 1/II trials of CART-BCMA in MM are ongoing (NCT03093168, NCT03070327, NCT02954445).

Although the results are promising, problems such as "on-target, off-tumor" toxicity and cytokine release syndrome (CRS) due to the CAR T cell infusion still exist (Dai, Wang et al.

2016). To overcome these problems, several approaches are possible. One is extensive screening of different CAR constructs on their on-tumor/off-tumor cytotoxicity. It has been shown that low affinity constructs can still mediate effective lysis of target cells as well as provide optimal proliferation and cytokine production (Drent, Themeli et al. 2017). Another approach is blocking the effects from IL-6, which play an important role in CRS, with an anti-IL6 receptor antibody for better management of CRS upon manifestation (Fitzgerald, Weiss et al. 2017).

1.5.6 Natural killer cell-based therapies

The progression of MM is associated with abnormal NK cell counts with a defective phenotype and decreased cytotoxic functionality. However, it has also been shown by others and us that *ex-vivo* expansion and long-term activation with addition of IL-2 and anti-CD3 mAb (OKT3) of autologous NK cells shows increased surface expression of activating receptors. Additionally, we also showed an elevated cytotoxicity against K562 target cells and autologous MM cells (Carlens, Gilljam et al. 2001, Alici, Sutlu et al. 2008) Although the exact role of OKT3 in this setting is unclear, it has been suggested that T cell activation leads to increased NK cell growth (Granzin, Wagner et al. 2017). Expanded donor derived NK cells from peripheral blood mononuclear cells (PBMCs) were used in a phase I clinical trial to treat five cancer patients. Infusion of the cells alone or in combination with subcutaneous IL-2 administration was save and no signs of acute graft versus host disease (GvHD) could be observed (Barkholt, Alici et al. 2009).

Several different expansion methods are currently used and tested which differ in start material (total PBMCs, CD3 depleted PBMCs, CD56 enriched NK cells, etc.), medium used, expansion system and many more factors. In general, it is possible to distinguish NK cell expansion in two groups: feeder-free and with addition of feeder cells. The use of feeder cells for large scale expansion has several advantages as they provide additional stimulation for activation and proliferation. Several sources of feeder cells can be used. These include irradiated cell lines which can be engineered to either express membrane bound cytokines or costimulatory receptors on their surface, as well as irradiated autologous and allogeneic PBMCs (Fujisaki, Kakuda et al. 2009, Lundqvist, Berg et al. 2011, Granzin, Soltenborn et al. 2015). However, contamination of residual feeder cells in the final product is a major concern for clinical applications and needs to be strictly controlled (Geraghty, Capes-Davis et al. 2014).

Especially the use of K562 cells that are transfected with membrane-bound (mb) IL-15 or IL-21 together with CD137L (41BBL) show a robust *ex-vivo* expansion and propagation of highly activated cytotoxic NK cells (Denman, Senyukov et al. 2012, Wang, Lee et al. 2013). Transfer of such expanded and activated NK cells into a murine MM model show persistence of NK cells up to four weeks together with inhibited MM growth (Garg, Szmania et al. 2012). Following this success, the same group tested those cells in seven heavily pretreated patients with high-risk relapsing myeloma. The cells were administered either fresh or cryopreserved and interestingly, *in-vivo* expansion could only be observed in the five patients that received

fresh cells. No serious adverse events related to treatment was observed. However, just two out of seven patients showed responses, one showed partial response and in the other the time of disease progression was decreased (Szmania, Lapteva et al. 2015).

In 2016, a phase I clinical trial (NCT02481934) was performed, where the combination of activated and expanded NK cells together with anti-myeloma drugs was tested in RRMM patients (Leivas, Perez-Martinez et al. 2016). NK cells were expanded for three weeks with K562-mbIL-15-41BBL feeder cells and then administered together with the pharmacological treatment. No major toxicities were reported, four out of five patients showed stabilization of the disease and two patients showed a 50% reduction in BM infiltration together with a response that lasted longer than one year. Infused cells are shown to have a highly cytotoxic phenotype with a high cytotoxicity *in-vitro*. Taken together, the *ex-vivo* expansion of autologous NK cells is feasible and these cells have an anti-tumor effect and multiple infusions are well tolerated in patients with relapsed or refractory myeloma.

The combination of an artificial and engineered receptor like CAR with highly cytotoxic T cells showed very promising results in several hematological malignancies (Haji-Fatahaliha, Hosseini et al. 2016). However, treatment with CAR T cells raises concerns based on their side effects in specific prolonged aplasia of the healthy parts of the target tumor, "off-tumor, on-target toxicity" and cytokine release syndrome together with a possible GvHD for allogeneic T cell products (Casucci, Nicolis di Robilant et al. 2013, Casucci, Hawkins et al. 2015).

To overcome these problems, utilizing NK cells with CARs could lead to similar functional outcomes without severe side effects. Innate immune cells recognize tumor cells based on pre-defined patterns and have previously been used in allogeneic and autologous settings without extreme side effects (Veluchamy, Kok et al. 2017). Additionally, the lifespan of mature NK cells in an adaptive setting is limited to a few days up to a few weeks and they do not induce GvHD in the recipient (Brehm, Huenecke et al. 2014, Glienke, Esser et al. 2015). One of the most significant limitations so far is the lack of persistence of NK cells in the allogeneic setting unless treatment is given to suppress rejection of the cells. Nevertheless, it has been shown in several studies that adoptive NK cell treatment has various advantages like the absence of life-threatening GvHD and major treatment-related toxicities (Miller, Soignier et al. 2005, Iliopoulou, Kountourakis et al. 2010, Rubnitz, Inaba et al. 2010, Bachanova, Cooley et al. 2014).

1.5.7 Natural killer cell lines in cancer therapy

The only NK cell line currently in use in the clinic is the NK cell line NK-92 that was isolated in 1992 from a 50-year-old male non-Hodgkin's lymphoma patient (Gong, Maki et al. 1994). NK-92 cells are known to be highly cytotoxic against target cells like K562 or primary tumor targets (Klingemann, Wong et al. 1996, Tonn, Becker et al. 2001). They express most of the activating NK cell markers but lack CD16 and the majority of the KIRs (only KIR2DL4 is expressed at low levels) and thus have an advantage over allogeneic NK cells as no MHC-class I restrictions apply (Maki, Klingemann et al. 2001). It is possible to grow NK-92

continuously in a feeder-free setting with the addition of IL-2 under current good manufacturing practices (CGMP) conditions to desired cell numbers for any possible treatment approach, which make them advantageous for an "off-the-shelf" immunotherapy product. Several clinical trials are currently exploring the possibility of using NK-92 for adoptive NK cell-based cancer immunotherapy (Suck, Odendahl et al. 2016).

When used for clinical trials, NK-92 are thawed from a master cell bank that has been tested extensively for possible contaminations with human pathogens like hepatitis C, human immunodeficiency virus, herpes simplex virus I and II, cytomegalovirus as well as traces of bacteria, fungi or mycoplasma (Tonn, Becker et al. 2001). The first study in which NK-92 cells were administered to patients already took place in 2001. In that study, NK-92 cells were irradiated prior to administration to 15 patients which suffered from high tumor load and had no other treatment options. Due to the bad shape the patients were in and the fact that all patients were immunocompromised, no efficacy could be evaluated. However, none of the patients suffered from severe side effects upon treatment with NK-92 independently of the infused dose (n = seven were given 1×10^9 cells/m^2 body surface, n = six were given 3×10^9 cells/m^2 body surface, and two patients received the maximum dose of 1×10^{10} cells/m^2 body surface). Another trial reported in 2008 that NK-92 cells in metastatic renal cell carcinoma and malignant melanoma showed very low infusion-related toxicities with some anti-tumor response despite the bad conditions of the patients (Arai, Meagher et al. 2008).

Recently, it was proven feasible to modify NK-92 cells with a CAR construct targeting either CS1 or CD138. Neither surface receptor of the modified cells, nor the specific lysis of human malignant plasma cells *ex-vivo* and in a xenograft mouse model were changed after this modification (Chu, Deng et al. 2014, Jiang, Zhang et al. 2014). Whether one should use a cell line or primary NK cells as source for CAR-modified NK cells to get an optimal product is yet in discussion (Tonn, Schwabe et al. 2013). Several clinical trials are currently run in Germany, Canada, China and the United States, which use NK cell lines to treat several hematological and solid tumors (Nowakowska, Romanski et al. 2018, Tang, Yang et al. 2018, Tomalka, Resto-Garay et al. 2018, Zhang, Zhang et al. 2018).

One of the arguments for using freshly isolated NK cells from peripheral blood is that they represent the most physiological source, although long-term cultivation and activation changes the receptor profile and could affect the functional competence of the cells. Another critical point is the possibility to achieve sufficient cell numbers for multiple infusions at high doses, especially for NK cells from immunocompromised patients. On the other hand, there is the immortalized cell line NK-92, a possible "off-the-shelf" product, with an inherent anti-tumor activity that can easily be modified genetically and banked. However, irradiation prior to infusion has an adverse effect on the *in-vivo* persistence of those cells and always carries a risk of not having a complete irradiation process (Tonn, Schwabe et al. 2013, Suck, Odendahl et al. 2016). Transduction of NK-92 cells with a CAR construct targeting CD138 led to enhanced cytotoxicity against $CD138^+$ expressing cell lines and primary MM cells compared to the unmodified NK-92 cells. Interestingly, irradiation of those cells with 10Gy, which is necessary to block proliferation, did not weaken the

cytotoxic response of the modified NK-92 cells (Jiang, Zhang et al. 2014). In 2016 a group, from Ohio showed that MM cancer stem-like cells express very high CS1 levels and would thus be an optimal target for potential CS1-CAR NK cells. Additionally, they observed that Dara triggered IFN-γ production in NK cells is both CD38 and CD16 dependent, which could be confirmed for both primary NK and $CD16^+$ NK-92 cells (Zhang, Chen et al. 2016). The combinatory targeting of CS1 together with CD38 through Dara could potentially have additive or synergistic effects to eradicate MM cells. This effect could be enhanced by the use of NK cells that are $CD38^-$, as this would prevent Dara-mediated fratricide. Long term effects and first clinical results are not published yet.
An overview of the different NK cell-based immunotherapy options is summarized in Figure 4.

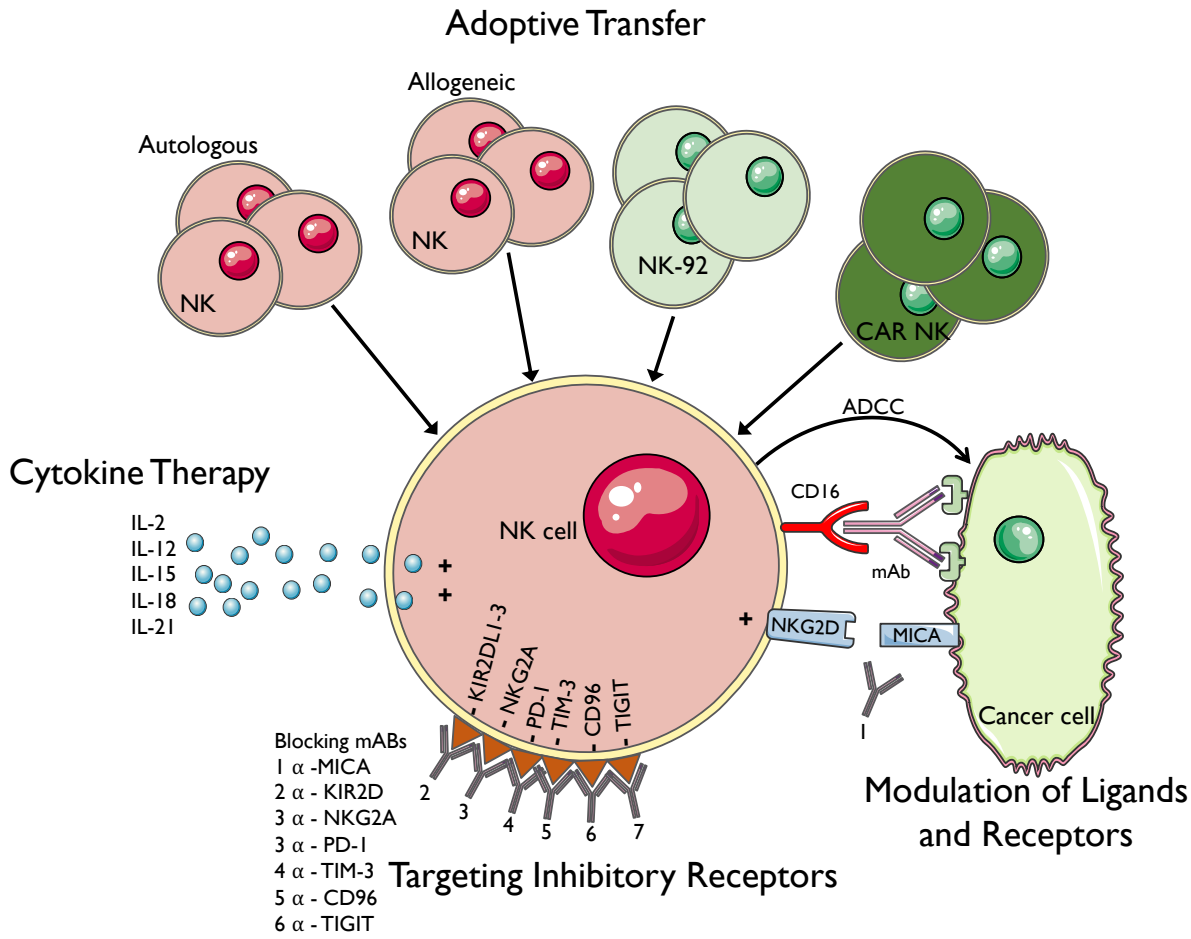

Figure 4: Overview of NK cell-related cancer immunotherapy options. CAR: chimeric antigen receptor; IL: interleukin; KIR: Killer cell immunoglobulin-like receptor; MICA: MHC class I polypeptide-related sequence A; NKG2A/D: natural killer group 2A/D; PD-1: programmed death-1; TIGIT: T cell immunoreceptor with Ig and ITIM domains; CD: cluster of differentiation; ADCC: antibody-dependent cellular cytotoxicity; mAb: monoclonal antibody; NK: natural killer.
Adapted from (Lorenzo-Herrero, Lopez-Soto et al. 2018)

2 GENERAL AIMS OF THIS THESIS

The first part of this thesis evolves around monoclonal antibody treatment in MM and its impact on the immune system of the patient. Being the first monoclonal antibody approved for MM treatment, Dara gives new hope for relapsed and refractory MM patients.

In study I we aimed to understand how treatment with Dara would affect NK and T cells in those patients, and also looked into *in-vitro* toxicity as well as a possibility to treat patients with Dara consecutively.

Following the findings in study I on the impact of NK cells in patients treated with Dara, we could then observe in study II that viral reactivations in patients treated with Dara were elevated despite antiviral prophylaxis. In study II we also compared clinical factors with immunological factors from flow cytometry-based phenotyping in order to see how the immune status of the patients changed with the administration of Dara. A lack of immune cells, especially NK cells, after Dara treatment, gives evidence that complementing adoptive cell therapy could have a positive impact on the disease progression in those patients.

In study III we assessed the possibility to grow the clinically relevant NK cell line NK-92 under serum-free conditions. It is known that serum has a big impact on expansion rates and phenotype, is also non-defined and has enormous batch to batch variations, which has a negative impact for clinical application. Creating a relatively cheap, reliable, xenofree culture system for NK-92 cells which retains their antitumor cytotoxicity would make an excellent "off-the-shelf" product for adoptive immunotherapy. This could potentially be complemented with other treatments such as monoclonal antibody therapy to achieve a synergistic effect.

3 RESULTS & DISCUSSION

3.1 STUDY I

Re-challenging with anti-CD38 monotherapy in triple-refractory multiple myeloma patients is a feasible and safe approach

Background

MM is a chronic disease for which there is no curative treatment. Known prognostic parameters that have a significant impact on OS are the treatment regimen, the patient's age, b2-microglobulin level and chromosomal aberrations (Harousseau, Avet-Loiseau et al. 2009, Biran, Jagannath et al. 2013, Iriuchishima, Saitoh et al. 2014, Blimark, Turesson et al. 2018). The type of clinical response (CR or PR) has also been shown to affect OS (Harousseau, Avet-Loiseau et al. 2009). During the last decades there have been considerable advances in the treatment outcome of MM.

At the moment, the only treatment able to cure MM is allogeneic stem cell transplantation for eligible patients under 70. Conventional therapy includes alkylating agents in combination with IMiDs and PIs. Nevertheless, OS is still just 13 months and event-free survival is at 5 months for patients that are double refractory to PIs and IMiDs (Weinhold, Ashby et al. 2016).

Dara, a monoclonal antibody targeting the CD38 epitope, which is highly expressed on malignant plasma cells, was approved for MM treatment by the U.S. FDA in 2015 and by the EMA in 2016 (Igarashi, Wynberg et al. 2004, Santonocito, Consoli et al. 2004, Lokhorst, Plesner et al. 2015). In RRMM, Dara is well tolerated and has demonstrated encouraging efficacy in MM patients (Plesner, Lokhorst et al. 2012). Together with Elo, Dara is the only monoclonal antibody for MM treatment with clinical approval; both can be used either as monotherapy or in combination treatment.

Aim of the study

In this study we investigated the feasibility of re-challenging two patients with Dara after they had progressed on this drug. We additionally monitored the immune status of those patients over the period of re-challenging to see the impact of the drug on the involved immune cells. Furthermore, we investigated the *in-vitro* toxicity of Dara on MM cells.

Results

We treated two patients suffering from triple refractory (IMiDs, PI and Cytostatics) MM with Dara as their last treatment option. Both patients had been treated with Dara before and had shown a good response before they consequently relapsed. Since both were eligible for a second round of Dara treatment, we initiated re-treatment with Dara to which both responded. Decreased M component levels could be observed for >7 months.

In order to see if this *in-vivo* effect could also be reproduced *in-vitro* and to understand the dose necessary for that, we incubated bone marrow cells before re-treatment with different doses of Dara. In the conditions where no Dara was added, we could observe a robust CD38 expression that was gradually decreased in a dose-dependent manner of Dara. Staining the samples with AnnexinV, which is an indicator for apoptosis, we could observe a direct dose-dependent effect of Dara on the MM cells and even a further increase of apoptosis when co-incubated with over-night IL-2 activated PBMCs, which underlines the ability of NK cells to kill the opsonized MM cells via ADCC.

We also assessed the phenotype of NK and T cells in the PBMCs and could see an immediate drop of NK cell counts after administration of Dara. Levels dropped within one week from 9% NK cells to around 2% in one of the patients and stayed that low during the whole treatment period. Furthermore, KIR phenotyping of circulating NK cells revealed a fluctuating KIR expression profile. $CD57^+$ NK cells transiently decreased during treatment and increased at each treatment interruption, which suggests a decrease in the circulating mature NK cell population during treatment. We could also observe this fluctuation in circulating $CD8^+$ T cells in patient one, where they completely disappeared after administration and reappeared during treatment interruptions. In addition, we also observed that during treatment interruption the number of anti-inflammatory myelomonocytes decreased drastically.

Significance

In this study, we could confirm that re-challenging triple refractory MM patients with Dara is possible and may offer new therapeutic treatment options as well as a possible synergy with adoptive NK cell treatment to maintain high levels of circulating NK cells.

MM patients that are refractory to IMiDs, PIs and cytostatic drugs and are not eligible for autologous stem cell transplantation because of their health status, age or lack of a suitable donor don't have any other treatment option left except the treatment with monoclonal antibodies like Dara or Elo. Dara, in general, is well tolerated and shows great single-agent activity, which leads to a positive response rate of about 36 % of all patients (Lokhorst, Laubach et al. 2014).

The key factor for a successful response to Dara treatment is high levels of CD38 surface expression on the MM cells. Nijhof et al. recently confirmed in a meta-analysis of two large

clinical trials that patients who achieved at least PR had a higher baseline CD38 expression compared to patients who did not achieve PR (Nijhof, Casneuf et al. 2016). However, the range of CD38 expression levels widely overlaps between responders and non-responders, thus a selection just by CD38 is not suitable.

As shown in this study, exposing Dara to MM cells *in-vitro* causes downregulation of CD38. This has also been confirmed by Nijhof et al. for several patients and various MM cell lines. MM cells were obtained before infusion, during treatment as well as at the progressive disease stage and CD38 levels were assessed respectively. Even after the first infusion, the surface expression of CD38 in the remaining MM cells was reduced by ~90%, as were the MM cell counts (Nijhof, Casneuf et al. 2016, Krejcik, Frerichs et al. 2017). The reduction of CD38 expression is a transient effect and is reversed after discontinuation of treatment with Dara (Nijhof, Casneuf et al. 2016). Several different mechanisms can lead to this reduction of CD38. After the direct response of the patient to Dara and the following elimination of MM cells with $CD38^{high}$ surface expression, only $CD38^{low}$ expressing cells remain. Also, internalization might also contribute to the loss of CD38. In addition, Horenstein et al. have published that CD38 molecules are able to cluster together and be released as tumor-derived microvesicles (Horenstein, Chillemi et al. 2015).

In this study, we showed for the first time that following initial Dara treatment at relapse, MM cells retained high levels of CD38 expression, which was later confirmed by others (Nijhof, Casneuf et al. 2016). Additionally, we could achieve PR over 6 months for patient 1 and >8 months for patient 2. These findings lead us to believe that retreatment with Dara may be a feasible approach independent of cytogenetic abnormalities.

One of the critical factors for re-treatment is the pool size of the effector cells exerting ADCC. NK cell-mediated killing of MM cells is an important aspect which determines the outcome of the response. It was shown that Dara-mediated ADCC is superior in patients with high NK cell to MM cell ratio, in comparison to a low NK cell to MM cell ratio; a similar effect could be observed for the frequency of activated circulating NK cells (Nijhof, Groen et al. 2015).

As shown in this study and by others, the administration of Dara will almost immediately lead to a depletion of NK cells. The underlying mechanisms could be the relatively high expression of CD38 on NK cells which could result in NK cell fratricide via ADCC. Interestingly, it was published by Wang et al. recently, that after Dara administration NK cells with a $CD38^{-/low}$ were enriched in treated patients, which strengthens the NK cell fratricide theory (Wang, Zhang et al. 2018). This in combination with the high numbers of anti-inflammatory myelomonocytes possibly leads to a decrease in ADCC-mediated anti-tumor activity that sustains during treatment.

While the amount of activated NK cells before treatment has a positive effect on the response, the combination of Dara with adoptive NK cell transfer could have very favorable synergies. An alternating treatment of Dara with NK cells could keep antibody levels high.

With each adoptive transfer, fresh activated NK cells would then be able to clear the remaining MM cells.

3.2 STUDY II

Infectious complications and NK cell depletion following daratumumab treatment of Multiple Myeloma

Background

MM is a plasma cell disease that is incurable, although new treatment methods like IMiDs or PIs have increased survival time drastically over the last decade. The monoclonal antibody Dara approved in 2015 by the U.S. FDA, is targeting CD38 on malignant plasma cells and shows very promising results in 36% of the patients (Lokhorst, Plesner et al. 2015). Additionally, it is the only single-agent treatment which shows the decrease of M-component, which is a surrogate marker for disease progression (Lokhorst, Plesner et al. 2015).

However, we and others have shown that lymphocyte counts, and especially NK cell counts, will drop after administering Dara, which leaves the patient at risk for viral reactivation or new infections with bacteria or viruses (Alici, Chrobok et al. 2016, Lonial, Weiss et al. 2016, Usmani, Weiss et al. 2016). Specifically, the numbers of $CD38^+$ NK cells will decline together with an oligoclonality of both $CD4^+$ and $CD8^+$ T cells which consequently leads to an impaired innate and adaptive immune system that is not able to maintain an effective antiviral defense (Mariani, Coscia et al. 2001, Krejcik, Casneuf et al. 2016).

It is known that infectious complications are one of the leading causes of mortality in MM patients, particularly herpes zoster infections and CMV (Marchesi, Mengarelli et al. 2014, Blimark, Holmberg et al. 2015, Hasegawa, Aisa et al. 2016). The cause of this is multifactorial and involves inherited immune defects, therapy-related weakening of the immune system and unknown factors due to the interaction of the patients' immune system with novel treatments like IMiDs (Teh, Harrison et al. 2016).

Aim of the study

In this study, we monitored the immune status of 23 patients that were included in a compassionate use program and treated with Dara. Nine out of the 23 suffered from either viral or bacterial infections or a combination of both. All patients had several lines of treatments before initiation of Dara as single-agent treatment. We observed clinical parameters as well as phenotypic markers, that were obtained by flow cytometry, in order to be able to find a link between immune status of the patient and infectious complication.

Results

The patients that were admitted for this study all had progressing MM and were heavily pretreated with multiple lines of treatment such as IMiDs, PIs, chemotherapy, allogeneic or autologous stem cell transplantation and did not have any alternative treatments left. Admission criteria of the patients were similar as for the phase II trial as described by Lonial *et al.* except for also excluding patients with a creatinine clearance <20ml/min (Lonial, Weiss et al. 2016).

The weekly Dara infusion schedule was at a starting dose of 16mg/kg body weight for eight weeks followed by a biweekly infusion for sixteen weeks and subsequent monthly infusions. Blood samples were taken prior to the first Dara infusion and successively after each following infusion if the health status of the patients allowed. Peripheral blood mononuclear cells were isolated and later analyzed by multicolor flow cytometry for NK and T cell subsets.

Out of the 23 treated patients, fourteen showed a response to treatment, which is comparable to previously described studies (Palumbo, Chanan-Khan et al. 2016). Although the response rate was high we could observe infections in nine patients, two from bacterial infections only, five from viral infections and two from both viral and bacterial infections. Interestingly, none of the patients that achieved complete remission suffered from any kind of infection.

As already described in study I, even in this study we could observe that NK cell counts dropped in all patients immediately after Dara infusion. This happened as fast as 24 hours after infusion and lasted over the time of Dara infusion. We could however not observe a recovery to normal NK cell levels (between 5 – 15 % of peripheral blood lymphocytes) in any of the patients. Another interesting observation was that the percentage of $NKG2A^{+}CD16^{+}$, which have a more immature phenotype, increased in some patients or were at least stable compared to the total lymphocyte count. Additionally, we followed the C-reactive protein (CRP) levels as well as total white blood cell counts (WBC) for all patients. CRP, which is a response marker for acute inflammation, correlates with the infection and is thus elevated. For some patients, we could observe a peak in CRP even before the infection was diagnosed which could have been caused by a bystander infection or an earlier infection/reactivation date that was clinically not yet linked to the infection.

Out of the 23 patients, two were included in a study where they received allo-SCT prior to Dara infusion. One of the two suffered from a varicella-zoster virus (VZV) reactivation seven weeks after the first dose of Dara was administered. Blood samples from this patient were not available at first Dara infusion, but from the time of sampling (week two) NK cell percentage was already low and decreased even further prior to the VZV reactivation. Interestingly, also the ratio of $CD4^{+}$ to $CD8^{+}$ T cells decreased in the weeks before the reactivation, which in general means that the patient has an accumulation of pro-inflammatory T cells and an impaired immune system.

The second patient did not suffer from any infection or latent reactivations during treatment but received two allo-SCT from a sibling donor. The WBC increased dramatically after the second allo-SCT together with the CRP. Remarkably, the distribution of $CD4^+$ to $CD8^+$ T cells inverted two times in correspondence to the two transplantations. Five weeks after the Dara infusion, the ratio decreased from 2.45 down to 0.44 and consequently increased again to reach 3.5 at ten weeks after the first infusion. It stayed at this high level even after the second transplantation and then reversed again 47 weeks after the first Dara infusion.

Significance

In study II we could show that patients who are treated with Dara have a higher risk to suffer from a primary viral or bacterial infection and that reactivation of latent viral infections is also increased. Due to the immunocompromised status of the patients, those infectious complications are potentially deadly. Close monitoring together with antiviral prophylaxis and adoptive NK cell therapy could overcome those problems.

The main cause of death for MM patients is infections. In a recent study it was shown that myeloma patients have a seven-fold higher risk for bacterial infection and a ten-fold higher risk for viral infections compared to sex- and age-matched healthy individuals; and 22% of deaths in MM patients are caused by infections (Blimark, Holmberg et al. 2015, Terpos, Kleber et al. 2015). To some extent, susceptibility to infections results from the myeloma itself, as the immune system is impaired due to B-cell dysfunction as well as functional abnormalities of dendritic, T and NK cells (Urashima, Ogata et al. 1996, Cook and Campbell 1999, Castriconi, Cantoni et al. 2003, Lee, Lee et al. 2004, Pinzon-Charry, Maxwell et al. 2005, Beyer, Kochanek et al. 2006, El-Sherbiny, Meade et al. 2007, Tete, Bijl et al. 2014). In addition, therapy-related factors can also play a role as well as age-related frailty and physical dysfunctions, making the patient more susceptible to infections (Nucci and Anaissie 2009, Kleber, Ihorst et al. 2013). Keeping the infectious complications in patients under control is key for longer survival and better treatment outcome.

Dara, which is an anti-CD38 humanized antibody, has direct and indirect effects on MM cells. One of the most important anti-tumor mechanisms is Dara-mediated ADCC. Once Dara binds to CD38 on the MM cells, NK cells are able to exert ADCC and lyse the tumor cells. NK cells, as well as many other cell types, express CD38 in low to intermediate levels, and upon activation CD38 expression is upregulated (Sconocchia, Titus et al. 1999, Funaro, Ferrero et al. 2000). However, we and others have published that CD38 expressing immune cells are depleted immediately after the first Dara infusion (Blimark, Holmberg et al. 2015, Alici, Chrobok et al. 2016, Krejcik, Casneuf et al. 2016, Casneuf, Xu et al. 2017).
NK cells are among the first responders to viral infections as well as to malignant transformation of cells. Dara-induced depletion leads to a lack of this cell population which is associated with repeated infections (Orange 2002). In this study, nine out of the 23 patients had infectious complication, five of which were reactivations with members of the herpesvirus family. In a recent publication, it was shown that after ASCT the rate of symptomatic CMV infection ranges between 0.7% to 30.7% (Marchesi, Pimpinelli et al. 2018).

Additionally, it is known that Bort as part of the MM treatment is associated with a higher incidence of CMV reactivation (Hasegawa, Aisa et al. 2016).

However, two big clinical trials (POLLUX & ALCYONE) comparing Dara treatment with len-dex and bort-melphalan-prednisone respectively could not observe any CMV infections (Dimopoulos, San-Miguel et al. 2018, Mateos, Dimopoulos et al. 2018).

Nevertheless, three patients in this study were identified for reactivation with CMV. In one of them, the infection was resolved without further treatment after a short Dara treatment interruption. It is not unlikely that CMV reactivation resolves itself in patients with hematological diseases unless they have undergone an allo-SCT (Styczynski 2018).

One patient, who was without antiviral prophylaxis, eventually died of a Herpes Simplex virus (HSV) reactivation, despite antiviral treatment. This was not seen in any of the trials before and might be due to recommended antiviral prophylaxis in RRMM patients. The use of prophylaxis could conceal the number of HSV or VZV reactivation in those patients. Immune response against HSV is complex and both the innate and adaptive immunes system are involved in controlling the infection. Especially the role of NK cells in HSV infections is controversial as discussed in several papers, and at least for mouse models it might depend heavily on the site of infection and the mouse strain (Habu, Akamatsu et al. 1984, Bukowski and Welsh 1986, Pereira, Scalzo et al. 2001, Dai and Caligiuri 2018).

Interestingly, we could observe that in three patients that showed reactivation from herpesviruses (CMV, HSV, VZV) the ratio between $CD4^+$ and $CD8^+$ T cells was very low. In a healthy individual, the ratio between CD4/CD8 ranges between 1.5 - 2.5 and a ratio >1.0 is associated with an impaired immune system due to chronic inflammation or infection (Bruno, Saracino et al. 2017). One possible explanation could be the depletion of $CD38^+$ T_{reg}s due to Dara-mediated ADCC which would reduce the total number of CD4 expressing T cells (Krejcik, Casneuf et al. 2016). Because of limitations in our flow cytometry panel, we could not assess $CD38^+$ T_{reg} counts and it is thus also just speculation if the ratio resulted from an expansion of $CD8^+$ or the depletion of $CD4^+$ cells.

Recent publications have found an adaptive subtype of NK cells, described as $CD57^+NKG2C^+$, which distinguishes them from regular NK cells. The presence of those cells, their rapid expansion and high specificity to the virus correlates with the CMV infection (Guma, Budt et al. 2006, Lopez-Verges, Milush et al. 2011, Hendricks, Balfour et al. 2014). Interestingly, in two out of three patients that showed CMV reactivation, we could not observe an increase in adaptive NK cells with the aforementioned phenotype.

In an 82y male, CMV reactivation could be detected just two weeks after the first Dara administration. This short time period together with the very low NK cell count after the first Dara infusion might be the reason why we could not observe any expansion of NK cells with an adaptive phenotype. This patient also had very high CRP levels directly before CMV reactivation and a slight decrease in mature NK cells that were able to exert ADCC ($CD16^+$ NK cells). In parallel, we could observe an increase from 25% to 45% of NK cells with a less

mature phenotype ($NKG2A^+CD16^-$). This shift from a more mature NK cell population to a less mature one was also observed by Rick Childs' group in an *in-vitro* experiment in 2015 (Cherkasova, Espinoza et al. 2015). When NK cells were sorted into $CD16^+$ and $CD16^-$ fractions by FACS, only $CD16^+$ NK cells were killed by Dara; no effect on $CD16^-$ population could be observed. Another recent case report by Frerichs *et al.* also reported a CMV reactivation in a heavily pretreated MM patient after Dara treatment. The reactivation is thought to have been caused by very low $CD4^+$ T cells counts as well as drastically reduced levels of B and NK cell levels, which might be a combinational result of pretreatment and Dara treatment (Frerichs, Bosman et al. 2019).

In this study, we could confirm what we published in study I: NK cells are specifically depleted upon Dara treatment. This results in a higher susceptibility for viral reactivations. As NK cells are one of the first-line defense mechanisms for infections, a synergistic adoptive NK cell therapy could lead to a better anti-myeloma treatment outcome and potentially reduce the risk for infections.

3.3 STUDY III

Functional Assessment for Clinical Use of Serum-Free Adapted NK-92 Cells

Background

Using adoptive cell transfer as a treatment for cancer has been practice since the first stem cell transplantations and has evolved dramatically since then. Especially in the last few years, more and more effort has been made in developing a more targeted therapy approach aiming at higher efficacy and fewer side effects and risks than the traditional treatments.

With the development of the CD19 CAR against B cell-derived malignancies, the combination of cell-based therapy and genetically modifying cells has been brought to the next level (Maude, Frey et al. 2014, Kochenderfer, Dudley et al. 2015). Not only several new targets are currently being developed to make use of those genetically modified cells, but also *ex-vivo* expanded or activated T or NK cells are widely being tested for several indications (Sutlu and Alici 2009, Dahlberg, Sarhan et al. 2015). One of the most pivotal components for successful cell expansion for clinical applications is human serum. All other components (media, cytokines, chemicals etc.) are available as chemically defined with a CGMP certificate. However, serum is also one of the key drivers for successful expansion and has a huge impact on fold expansion or composition of the final cell product. The other critical part is the source of the cells; allogeneic and autologous cells both have their advantages and disadvantages for certain cancer indications.

The uses of irradiated cytotoxic NK cell line for adoptive therapy has been explored by several groups and so far, the results have been promising (Gong, Maki et al. 1994, Arai, Meagher et al. 2008, Tonn, Schwabe et al. 2013, Nowakowska, Romanski et al. 2018). When trying to create an "off-the-shelf" product, serum dependency is still a big hurdle and cells grown under fully defined conditions would have a more predictable outcome in terms of cell proliferation, cytotoxicity and phenotype.

Aim of the study

In this study, we investigated if NK-92 cells can be grown serum free with inherited phenotype and antitumor responses. Critical steps included culturing these cells for long term and being able to repeatedly freeze and thaw them. Additionally, we compared the transcription profile of serum free and serum complemented cells and explored susceptibility to genetic modifications.

Results

We gradually decreased the serum concentration from initially 20% to 0% in the culture conditions over the course of three weeks, followed by a three-week recovery period. While NK-92 grow in spheres in regular culture conditions, they lose this growing habit once the serum is gradually reduced, probably due to stress by the low serum levels. After a short three-week recovery period the cells grow at a comparable tempo and also spheres can be observed again. We could show that long term culture, up to several months, under serum-free conditions is possible and that serum-free NK-92 cells don't show higher apoptotic cell percentage during culturing. In order to be able to compare the cells from both culture conditions, we assessed the phenotype of the activating and adhesion receptors in a flow-based assay. We could not determine any significant difference. Although the phenotype wasn't altered, we could observe a decrease in functionality in a standard four-hour chromium release assay as well as in a degranulation assay against the target cell line K562.

The load of the NK-92 cells with the cytotoxic molecules granzyme A & B and perforin was comparable although the total load varied between different batches. However, if those cells were used for a therapeutic approach they would be infused to a patient and by that have contact with serum again. To simulate this condition, we added serum to the serum-free culture for 16 hours and performed the same functional assays. We could then observe that the decreased cell lysis of K562 cells was reversed and comparable to serum-supplemented culture.

To get better insights into the biological mechanism that plays a role in adaption to serum-free culture conditions we performed RNA sequencing. We observed that especially the genes of the antigen presenting pathway were upregulated in serum-free cultured NK-92 cells. Also, genes related to MHC class I, as well as interferon-stimulated genes and MYC were elevated. Interestingly, the change in expression levels seems to be most prominent in the step between 5% to 0% serum. Lastly, we also showed that repeated freeze and thaw cycles are well tolerated by serum-free cultured NK-92 cells without an increase in apoptosis or decrease in functionality.

Significance

In this study, we adapted NK-92 cells to long term serum-free culture conditions which showed an inherited phenotype and comparable doubling times. Although functionality towards K562 is decreased under serum-free conditions, we could observe that reintroduction of serum reverses the effect and leads to equivalent killing, compared to serum-cultured NK-92. This culturing method is an affordable expansion procedure for clinically grade NK-92 cells and is as feasible as current standard clinical manufacturing protocols.

NK cells obtained from patients with progressing cancer are mostly hypo-responsive toward the autologous tumor cells. If those primary NK cells are expanded and activated *ex-vivo* in the absence of the tumor, their functionality and ability to recognize autologous tumor cells can be restored. One of the biggest hurdles is achieving sufficient cell numbers with the desired phenotype. Not all expansion yields cell numbers that are necessary for clinical application and this not only depends on the cell source but largely on the serum. As neither human serum nor fetal calf serum is chemically defined and can vary widely in the levels of hormones, lipids, and proteins, the impact on a particular cell type might be enormous. Finding the right serum for one specific cell type and one specific way of culturing cells can be time-consuming and costly. For example, a growth factor for one cell type could cause differentiation in another cell type; or the exposure of female-derived PBMCs to testosterone could induce dramatic functional changes (Moscovis, Cox et al. 2015).

Lately, research groups have focused more and more on the impact of extracellular vehicles (EVs) and their impact on cell to cell communications. Among other functions, EVs can be internalized by cells and deliver their cargo such as mRNA, miRNA, and rRNA and thus have a direct impact on intercellular signaling (Shelke, Lasser et al. 2014). Serum contains large amounts of EVs, which have a significant impact on the growth and behavior of cultured cells (Eitan, Zhang et al. 2015, Wei, Batagov et al. 2016). Although sera from several donors are pooled together during clinical grade serum production to minimize the impact of each component, variation between each batch is high and has a direct impact on the final cell product.

Reproducing results and achieving a consistent product across research groups and production facilities is difficult to accomplish. This is due in part to differences in assay protocols, cell models or just equipment, which can be changed and optimized, but also due to undefined components such as serum. It is not only necessary to conduct a pre-screening of several different batches but also to acquire large enough batch sizes for long term studies, which is challenging. All in all, a fully-defined cell expansion system is in many factors superior compared to a system that heavily relies on non-defined components such as serum.

Using a cell line for therapy has the advantage that the production is scalable and predictable numbers of highly cytotoxic cells are easily obtained without a biological donor variation. As described in the study, it could be observed that serum-free cultured NK-92 cells show a lower cytotoxic capacity, but we could also show that over-night exposure to serum could restore the cytotoxicity of the cells. This reintroduction would mimic a possible infusion scenario where bedside thawed, irradiated serum-free grown NK-92 were administered into a patient and would thus come into contact with serum, which might boost their cytotoxic capacity.

Several clinical trials are currently ongoing, which use the NK-92 cell line either just activated and unmodified or modified. One way of modification is using a CAR e.g. against HER2, CD19 another option is expressing CD16 so that the combination with a mAb treatment is possible (Nowakowska, Romanski et al. 2018, Tang, Yang et al. 2018, Tomalka,

Resto-Garay et al. 2018, Zhang, Zhang et al. 2018). The following studies are currently recruiting for either solid tumors or hematological malignancies: NCT03383978, NCT03656705, NCT03027128, NCT02892695, NCT03563170, NCT03586869 (from clinicaltrials.gov); several more have also already been announced.

With the culture method described in study III we could show that NK-92 cells are able to grow under serum-free, chemically-defined media conditions with only the addition of IL-2. Adapting this robust and affordable expansion procedure for future clinical trials could decrease manufacturing costs and eliminate the variation of serum batch to batch variation.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVE

With the clinical approval of the first CAR T cells products, YESCARTA and KYMRIAH in 2017 and 2018 respectively, and remarkable results in B cell lymphoma, immunotherapy moved into the focus of not only scientists and clinicians but also the general public.

Dara, as the first approved mAb for MM treatment, had breakthrough success and gave new hope to relapsed/refractory patients. Prolongation of life and better disease control even in heavily pretreated patients are key features of this treatment. Over the last decade countless research groups helped understand the impact of the immune system in MM and specifically the role of NK cells. We and others showed that *ex-vivo* expanded NK cells have significant cytotoxicity against autologous MM cells without targeting healthy tissue. These findings make NK cells an optimal source for NK cell-based adoptive therapy. Several trials have explored this treatment option with NK cells from various sources.

However, with the first Dara treated patients it has become clear that despite the huge treatment related success there are also drawbacks and side effects with this treatment. NK cells and partly other CD38 expressing immune cells are depleted after Dara administration, which can lead to an increased risk for viral and bacterial infections. This is especially important because one of Dara's main mechanisms of action is NK cell-mediated ADCC. The reduced pool of effective NK cells might lead to a possible reduction of Dara effectiveness.

Combining Dara treatment with adoptive NK cell treatment could have a positive synergistic effect on MM disease control. Alternating infusing expanded, activated NK cells with Dara, would keep cytotoxic levels of NK cells high and boost the effect Dara has on fighting MM.

Various sources can be used to obtain sufficient numbers of highly active NK cells. Using a cell line which combines characteristics of an NK cell (cytotoxicity and cytokine production) with that of a tumor cell (limitless proliferation potential) has the advantage of predictable, functional active, "off-the-shelf" cells being easily producible.

For streamlined NK cell production, biological variation and undefined expansion components are problematic. Adapting NK-92 as a model cell line, and later primary NK cells to serum-free culture conditions, eliminates the batch to batch variation of the chemically undefined serum. Combining a defined, robust expansion protocol to obtain clinical grade NK cells for adoptive therapy in combination with mAb treatment could be a breakthrough for future treatment regimens.

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